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<p>(54) Title: COMPLEXES OF MODIFIED LYMPHOTOXINS AS PHARMACEUTICAL PREPARATIONS</p> <p>(57) Abstract</p> <p>This invention relates to lymphotoxin (LT) complexes comprising lymphotoxin-<math>\alpha</math> (LT-<math>\alpha</math>) and lymphotoxin-<math>\beta</math> (LT-<math>\beta</math>) subunits, and modified versions thereof, which can act as specific inhibitors of the biological events mediated by the ligands and receptors of the tumor necrosis factor (TNF) family. This invention also relates to unique portions of the LT-<math>\alpha</math> and LT-<math>\beta</math> protein sequences, "LT subunit association domains", which potentiate subunit assembly into an active trimeric ligand. This invention provides TNF-related ligand monomers mutated in their respective subunit association domains which permits them to form heteromeric complexes with LT subunits. Altered ligands which have only one functional receptor binding site per heteromer can inhibit signalling by that receptor. Also provided are mutant and chimeric LT subunits with can alter the receptor binding properties of heteromeric complexes assembled from them. Polypeptides comprising LT subunit association domains, LT heteromeric complexes which inhibit receptor signalling, pharmaceutical compositions comprising LT heteromeric inhibitors, and methods for treatment using those pharmaceutical compositions are also provided.</p>			

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**COMPLEXES OF MODIFIED LYMPHOTOXINS  
AS PHARMACEUTICAL PREPARATIONS**

**TECHNICAL FIELD OF THE INVENTION**

This invention relates to lymphotoxin (LT)

- 5 complexes comprising lymphotoxin- $\alpha$  (LT- $\alpha$ ) and lymphotoxin- $\beta$  (LT- $\beta$ ) subunits, and modified versions thereof, which can act as specific inhibitors of the biological events mediated by the ligands and receptors of the tumor necrosis factor (TNF) family. This
- 10 invention also relates to unique portions of the LT- $\alpha$  and LT- $\beta$  protein sequences, "LT subunit association domains", which potentiate subunit assembly into an active trimeric ligand. This invention provides TNF-related ligand monomers mutated in their respective
- 15 subunit association domains which permits them to form heteromeric complexes with LT subunits. Altered ligands which have only one functional receptor binding site per heteromer can inhibit signalling by that receptor. Also provided are mutant and chimeric LT
- 20 subunits with can alter the receptor binding properties of heteromeric complexes assembled from them.
- Polypeptides comprising LT subunit association domains, LT heteromeric complexes which inhibit receptor signalling, pharmaceutical compositions comprising LT
- 25 heteromeric inhibitors, and methods for treatment using those pharmaceutical compositions are also provided.

- 2 -

BACKGROUND OF THE INVENTION

Tumor Necrosis Factor (TNF)-related cytokines have emerged as a large family of pleiotropic mediators of host defense and immune regulation. Members of this 5 family exist in membrane-bound forms which act locally through cell-cell contact, or as secreted proteins which can act on distant targets. A parallel family of TNF-related receptors react with these molecules and trigger a variety of pathways including cell death, 10 cell proliferation, tissue differentiation and proinflammatory responses.

Defined originally as a pair of genes in 1984, the TNF family of ligands and receptors has grown to at least 9 receptor-ligand pairs (Smith et al., 15 Cell, 76, pp. 959-62 (1994)). These ligand-receptor pairs (L::R) include: TNF::TNF-R, LT- $\alpha$ ::TNF-R, LT- $\alpha$ / $\beta$ ::LT- $\beta$ -R, FasL::Fas, CD40L::CD40R, CD30L::CD30, CD27L::CD27, Ox40L::Ox40 and 4-1BBL::4-1BB.

TNF and LT- $\alpha$  are cytokines with an 20 extraordinary range of activities mediated by TNF receptor activation. There are two TNF and LT- $\alpha$  specific receptors, called herein "TNF-R", which are elsewhere referred to as p55 (or p60), and p75 (or p80) TNF-R. TNF-R activation is associated with immune and 25 inflammatory reactions, septic shock, autoimmune disorders and graft-host disease (Beutler, B., Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine. New York: Raven Press (1994)). It would be useful in many instances to treat the 30 reactions and disorders associated with TNF-R by selectively inhibiting TNF-R signalling.

A third receptor binds LT heteromeric complexes (predominantly LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1) but does not bind TNF or LT- $\alpha$  (Crowe et al., Science, 264, 35 pp. 707-10 (1994)). This third receptor is called

- 3 -

herein the LT- $\beta$  receptor ("LT- $\beta$ -R"). LT- $\beta$ -R signalling may play a role in peripheral lymphoid organ development, and may also play a role in humoral immune responses. The ability to selectively block the LT- $\beta$ -R pathway would also be useful.

- Another member of the TNF superfamily is the FasL::Fas complex. Signalling through the Fas pathway also has pleiotropic effects. Activated Fas can kill cells by triggering apoptosis -- programmed cell death.
- 5 The Fas pathway represents a major mechanism for cytolytic T lymphocyte killing (Lowin et al. Nature, 370, pp. 650-52 (1994); Kojima et al. Immunity, 1, pp. 357-64 (1994)). Recent studies in mice suggest that Fas signalling is involved in apoptosis of chronically-
- 10 activated mature T cells, and suggest a role for Fas signalling in peripheral tolerance (Russel and Wang, Eur. J. Immunol., 23, pp. 2379-82 (1993)). Fas-induced cell death may also a major component of graft rejection. Inhibitors of the Fas pathway may be useful
- 15 in modulating activated T cell apoptosis and in reducing tissue rejection following organ transplantation. In addition, signalling through the Fas receptor is thought to be involved in liver necrosis during fulminant hepatitis (J. Ogasawara et al., Nature, 364, pp. 806-80, (1993)). Inhibitors of the Fas pathway may also be useful in blocking HIV-induced lymphocyte depletion (Katsikis et al., J. Exp. Med., 181, pp. 2029-36 (1995)). The ability to block this signalling pathway would be beneficial in such
- 20 instances.

In contrast, some primary B thymocytes (PBTs) and B cell tumors respond to Fas activation by proliferating rather than by apoptotic death (Mapara et

- 4 -

al., Eur. J. Immunol., 23, pp. 702-8 (1993); Alderson et al., J. Exp. Med., 178, pp. 2231-35 (1993)). In these instances, inhibition of Fas signalling could be useful in diminishing deleterious B cell proliferation.

5 Signalling through the CD40R, another TNF family member, initiates immunoglobulin (Ig) maturation and secretion. Immature B cells make only surface IgM. When CD40R on an immature B cell binds to CD40L on the surface of an activated CD4<sup>+</sup> helper T cell, CD40  
10 signalling stimulates the B cell to undergo Ig isotype switching and to secrete mature Igs. B cells proliferate and mature into memory and antibody-secreting cells. This role for CD40R signalling has been confirmed in humans. Patients who harbor  
15 mutations in their CD40L gene can make only IgM antibodies and are severely immunodeficient (Callard et al., Immunol. Today, 564, pp. 559-64 (1993)).

Specific inhibitors of the CD40 signalling pathway could be useful in suppressing undesirable B  
20 cell proliferation and isotype switching. In fact, inhibitors of the mouse CD40 pathway block antibody responses and autoimmune disease development in mice (Durie, F. H. et al., Science, 261, pp. 1328-133 (1993)), and HIV proliferation *in vitro* (Pinchuk et  
25 al., Immunity, 1, p. 317 (1994)).

Other TNF family members, including CD27, CD30, 4-1BB and OX-40, appear to play roles in T cell proliferation and cytokine regulation. Recent data suggest that the 4-1BB pathway plays a role in  
30 costimulation of IL-2 production and in inducing T cell proliferation (DeBenedette et al., J. Exp. Med., 181, pp. 985-92 (1995)). The OX-40 cell surface antigen is a marker of activated CD4<sup>+</sup> T cells that acts as a costimulatory receptor of CD4<sup>+</sup> T cell proliferation

- 5 -

(W.R. Godfrey et al., J. Exp. Med., 180, pp. 757-62 (1994)).

The ability to selectively inhibit signalling through these pathways may also be useful in regulating 5 certain cytokine pathways or in controlling unwanted T cell proliferation. Inhibitors of these other TNF-like receptor pathways will be especially useful in the future when the players and events mediated by these receptors are more clearly understood.

10 TNF-R signalling is activated through the close spatial aggregation of multiple receptors on the cell surface, a process interchangeably called "receptor clustering" or "receptor cross-linking". TNF-R receptor clustering is mediated by its 15 specificity for multivalent ligands -- TNF or LT- $\alpha$ 3. Each trimeric ligand binds to and clusters up to three TNF-R molecules. TNF-R clustering and activation can also be induced using receptor-specific antibodies (Engelmann et al., J. Biol. Chem., 265, pp. 14497-504 20 (1990)). Fas (Ogasawara et al., Nature, 364, pp. 806-809 (1993)) and LT- $\beta$ -R signalling (applicants' co-pending United States application Serial No. 08/378,968) can also be activated by receptor cross-linking using appropriate receptor-specific antibodies.

25 It would be useful to selectively inhibit signalling through TNF-R, LT- $\beta$ -R, Fas, CD40R, and other TNF-like receptors to regulate aberrant signalling or to modulate undesirable effects caused by receptor signalling in patients with imbalances or disorders in 30 immune reactions such as those described above. One way to achieve selective inhibition would be to modify TNF or a TNF-like ligand so that it could bind to its cognate receptor but could not cluster adjacent receptors. A TNF-related multivalent ligand altered to

- 6 -

bind only one surface receptor molecule would inhibit receptor signalling by blocking aggregation of adjacent surface receptor molecules. LT- $\alpha$ /LT- $\beta$  heteromeric ligands constitute such inhibitors and form the basis  
5 of this invention.

SUMMARY OF THE INVENTION

The present invention solves the problems referred to above by providing pharmaceutical compositions and methods for inhibiting TNF and TNF-related receptor signalling by inhibiting cell surface receptor clustering. In one embodiment of this invention, lymphotoxin complexes formed between LT- $\alpha$  and LT- $\beta$  subunits are provided ("LT- $\alpha$ / $\beta$  heteromeric complexes") which inhibit TNF-R signalling. Preferred compositions and methods of this embodiment comprise soluble LT- $\alpha$ 2/ $\beta$ 1 trimers. In a more preferred embodiment, defined residues of the LT subunits are mutated to eliminate any low level binding activity to LT- $\beta$ -R, and if desired, to increase the relative  
10 15 20 binding affinity of the LT heterotrimer for either p55 or p75 TNF-R.

In another embodiment of this invention, LT- $\alpha$ / $\beta$  heteromeric complexes which inhibit LT- $\beta$ -R signalling are provided. Preferred compositions and methods of this embodiment comprise soluble LT- $\alpha$ 1/ $\beta$ 2 trimers, and soluble LT- $\alpha$ 2/ $\beta$ 1 trimers which are mutated at defined residues within LT- $\alpha$ -containing clefts to reduce or eliminate binding to TNF-R. The altered LT- $\alpha$ 2/ $\beta$ 1 heterotrimer is a specific inhibitor of LT- $\beta$ -R  
25 30 signalling.

In a more preferred embodiment, specific residues which mediate receptor binding are mutated to selectively increase or decrease the binding affinity of the LT heterotrimeric inhibitor for LT- $\beta$ -R. In

- 7 -

another more preferred embodiment, both sets of mutations are incorporated into the trimeric inhibitors to reduce background binding and to modulate inhibitor binding to the chosen targeted receptor.

5        In another embodiment of this invention, the LT trimeric scaffold structure is used as a base from which other TNF-like ligand receptor binding domains are displayed to make a chimeric LT inhibitor with altered receptor binding characteristics. In one  
10      preferred embodiment, residues in the LT- $\alpha/\alpha$  cleft of a soluble LT- $\alpha_2/\beta_1$  trimer required for TNF-R binding are exchanged with a corresponding portion of the receptor binding domain of a heterologous TNF-like ligand to target the soluble trimeric inhibitor to that other  
15      TNF-like receptor. In another preferred embodiment, similar changes are made to residues in the LT- $\beta/\beta$  cleft of a soluble LT- $\alpha_1/\beta_2$  trimer to convert that cleft into a receptor binding site of a different TNF-like receptor. In more preferred embodiments,  
20      mutations which reduce background binding of the soluble chimeric LT inhibitors to LT or TNF receptors are also incorporated. Pharmaceutical compositions and methods using these complexes are provided.

In a further embodiment of this invention, an  
25      inhibitor of a TNF-like receptor is made by altering a corresponding TNF-like ligand subunit in its subunit association domain so that it can form mixed heterotrimers with LT subunits. In one preferred embodiment, a trimeric inhibitor of a TNF-like receptor  
30      is formed by changing residues of the TNF-like subunit association domain to more closely resemble those of an LT subunit association domain. In another preferred embodiment, a LT subunit association domain is grafted onto a TNF-like ligand subunit (or variant or portion  
35      thereof) comprising a receptor binding domain in a

- 8 -

position which does not significantly disrupt the ability of the TNF-like receptor binding domain to interact with its cognate receptor.

**BRIEF DESCRIPTION OF THE DRAWING**

5      **Figure 1.** Alignment of the extracellular domains of five members of the TNF family of ligands: LT- $\alpha$ , LT- $\beta$ , TNF, CD40L and FasL with a referenced numbering system used herein. LT- $\alpha$  residues involved in trimerization are underlined and define the LT- $\alpha$  subunit association  
10 domain.

**Figure 2.** Purification schemes: various combinations of TNF or LT receptor affinity columns can be used to purify LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1.

15      **Figure 3.** Analysis of individual LT multimer fractions analyzed by C4 reverse phase HPLC show the relative ratios of LT- $\alpha$  and LT- $\beta$  in each preparation.

20      **Figure 4a.** Gel exclusion sizing analysis of the LT trimers purified with receptor affinity columns. Pure proteins were analyzed on a TSK 3000 HPLC resin in a phosphate buffered saline buffer.

**Figure 4b.** Ion exchange chromatographic analysis of the LT trimers purified with receptor affinity columns.

25      **Figure 5a.** Biacore™ sensorgrams of the binding of LT- $\alpha$ 3, LT- $\alpha$ 2/ $\beta$ 1 and LT- $\alpha$ 1/ $\beta$ 2 to the p55 TNF receptor as a function of time. Various concentrations of the ligand are flowed across a Biacore™ chip and the association and dissociation phases of ligand binding are monitored.

30      **Figure 5b.** Biacore™ sensorgrams of the binding of LT- $\alpha$ 3, LT- $\alpha$ 2/ $\beta$ 1 and LT- $\alpha$ 1/ $\beta$ 2 to the LT- $\beta$  receptor as a function of time, as described in **Figure 5a**. **Figure 5b** shows that both LT- $\alpha$ 2/ $\beta$ 1 and LT- $\alpha$ 1/ $\beta$ 2 bind well to LT- $\beta$ -R, but that LT- $\alpha$ 2/ $\beta$ 1 comes off more quickly than LT- $\alpha$ 1/ $\beta$ 2 and thus has a lower affinity for LT- $\beta$ -R.

- 9 -

- Figure 6.** Ability of soluble LT- $\alpha$ 2/ $\beta$ 1 complexes to block the anti-proliferative activity of TNF on: (a) a mouse indicator line L929, (b) a human cervical carcinoma ME180 in the presence of IFN- $\gamma$ , and (c) a 5 human colon carcinoma HT29 in the presence of IFN- $\gamma$ . In each case the concentration of LT- $\alpha$ 2/ $\beta$ 1 is varied in the presence or absence of 1 ng/ml r-hu-TNF. The dashed lines indicate the absorbance values obtained for cells grown in the absence of TNF (top dashed line) 10 or the presence of 1 ng/ml TNF (bottom dashed line).
- Figure 7.** Ability of soluble LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1 to block the binding of a soluble form of the LT- $\beta$ -R to the LT- $\alpha$ / $\beta$  ligand on a cell surface thus mimicking how a LT- $\alpha$ 1/ $\beta$ 2 complex would block LT- $\beta$ -R signalling. LT- 15  $\alpha$ 1/ $\beta$ 2 or LT- $\alpha$ 2/ $\beta$ 1 were added to PMA-activated II-23 cells and then soluble LT- $\beta$ -R-Fc was allowed to bind to the surface ligand. Bound human Fc was quantitated using anti-human IgG-phycoerythrin labelled antibodies and FACS analysis was performed.
- Figure 8.** Comparison of the cytotoxic effects of wild type LT- $\alpha$ 1/ $\beta$ 2 and a LT- $\alpha$ (D50N)1/ $\beta$ 2 mutant on growth of: (a) mouse WEHI 164 cells, and (b) a human colon carcinoma tumor line HT29. Growth was quantitated after 3 days by measuring the optical density (OD 550) 20 of reacted MTT, which is proportional to cell number.
- Figure 9.** The structure of the alpha carbon backbone of LT- $\alpha$ , illustrating the relatively few amino acid residues involved in trimerization. Those residues critical to the formation of a trimeric structure are 25 highlighted.
- Figure 10.** The amino acid sequences of CD40L, FasL and TNF C-terminal extracellular domains altered to drive trimerization with either wild type LT- $\beta$  (for LT- $\alpha$  chimeras) or with wild type LT- $\alpha$  (for LT- $\beta$  chimeras). 30 Underlined residues represent sequences from LT- $\alpha$  or LT- $\beta$  subunit association domains, respectively.

- 10 -

**DETAILED DESCRIPTION OF THE INVENTION**

The following detailed description is set forth so that the invention herein described may be fully understood. It is useful to define the following  
5 terms so that the description may be more easily understood.

The term "apoptosis" refers to a process of programmed cell death.

The term "association" refers to a physically  
10 detectable condition of proximity between a portion of one ligand subunit with a portion of the same or a different ligand subunit wherein the juxtaposition is energetically favored by electrostatic, van der Waals or covalent interactions. A substantial disruption of  
15 the association between subunits is one which can be detected by a variety of methods.

The terms "association sequence" and "association domain" both refer to a region (or portion thereof) of a LT polypeptide which drives the  
20 association between itself and other LT subunit association sequences to form the core scaffold structure of a LT multimeric ligand complex. LT association domains typically comprise a  $\beta$ -sandwich structure and typically self-assemble into a trimeric  
25 scaffold structure.

The terms "backbone" and "scaffold" interchangeably refer to the internal  $\beta$ -sandwich structure formed between multiple LT monomers oligomerizing through their subunit association  
30 domains.

The term " $\beta$ -pleated sheet" refers to a sheetlike secondary structure assumed by portions of a polypeptide chain, the sheet held together by hydrogen bonds. The term " $\beta$ -sandwich" refers to a structure  
35 comprising two antiparallel  $\beta$ -pleated sheets with a

- 11 -

"jelly-roll" or Greek key topology (Jones et al., 1989; Eck et al., 1992).

The term "complex" refers to one monomeric polypeptide subunit associated with at least one other 5 polypeptide subunit. When subunits of the same type ("like subunits") associate, a "homomeric complex" is formed. When one or more like subunits associate with at least one different type of subunit, a "heteromeric complex" is formed. The subunits can associate through 10 electrostatic, van der Waals, or covalent interactions.

The term "cytokine" refers to a protein released by one cell type or population which acts on at least one other cell type or population to mediate intercellular signalling. Cytokine receptors bind 15 cytokines and can trigger signalling events within the cell. The TNF family of ligands are one set of many proteins that fall within the generic class of cytokines. Other cytokines include hormones, growth factors, inductive factors, stimulating factors, 20 inhibiting factors, interleukins, integrins, interferons and other polypeptide factors.

The term "immunological tag" refers to a portion of a polypeptide chain comprising an epitope capable of interacting with an antibody.

25 The term "lymphotoxin (LT)" refers to a member of the TNF-related cytokine family which is produced by activated T cells. The original lymphotoxin- $\alpha$  (LT- $\alpha$ , previously called TNF- $\beta$ ) and a newer member, lymphotoxin- $\beta$  (LT- $\beta$ ), comprise a system 30 of secreted and membrane-anchored regulatory molecules involved in immunoregulation and host defense. LT subunits typically form homotrimers or mixed heterotrimers. These complexes are described in detail in applicants' co-pending international application 35 (PCT/US93/11669, published January 9, 1992 as WO 94/13808), which is herein incorporated by reference.

- 12 -

The terms "LT- $\alpha$  mutein" and "LT- $\beta$  mutein" refer to LT- $\alpha$  or LT- $\beta$  polypeptides having one or more amino acid changes or mutations compared to the amino acid sequence of the corresponding native polypeptide.

5       The terms "LT- $\alpha$  biological activity", "LT- $\beta$  biological activity", and "LT- $\alpha/\beta$  biological activity" are defined as: 1) immunological cross-reactivity with an antibody directed against at least one epitope of the corresponding native subunit or complex of  
10 subunits; or 2) the ability of the subunit or complex of subunits to compete for ligand binding sites on a LT-specific receptor such as TNF-R or LT- $\beta$ -R; or 3) the ability to stimulate an immune regulatory response or cytotoxic activity qualitatively in common with a  
15 native LT subunit or complex.

The term "LT- $\alpha/\beta$  heteromeric complex" refers to an association between at least one LT- $\alpha$  subunit and at least one LT- $\beta$  subunit. Preferably, the LT- $\alpha/\beta$  heteromeric complex has no more than two adjacent  
20 subunits comprising a binding site for a single receptor molecule. Most preferably, the complex has the stoichiometry LT- $\alpha 1/\beta 2$  or LT- $\alpha 2/\beta 1$ , and includes soluble, altered and chimeric forms thereof. Soluble LT heteromeric complexes lack a transmembrane domain  
25 and can be secreted by an appropriate host cell which has been engineered to express LT- $\alpha$  and/or LT- $\beta$  subunits. Examples of soluble LT heteromeric complexes are described in applicants' co-pending international application (PCT/US93/11669, published January 9, 1992  
30 as WO 94/13808).

The term "LT heterotrimeric complex" refers to a LT heteromeric complex having three subunits, at least one of which has a portion of a LT subunit association domain or a portion of a LT receptor  
35 binding domain (TNF-R or LT- $\beta$ -R).

- 13 -

An "altered" or "modified LT heteromeric (or heterotrimeric) complex" is a multimeric ligand whose scaffold comprises more than one type of LT or LT chimeric subunit and in which at least one region of at 5 least one of the LT or LT chimeric subunits has been modified, typically by mutation, to alter the ultimate receptor binding characteristics of the LT heteromeric complex.

The terms LT "chimeric heteromer", "chimeric 10 heterotrimer" and "chimeric ligand" all refer to a heteromeric ligand comprising subunits each having a LT or LT-like subunit association domain which drives heteromer formation, and having an appropriate number of subunits with altered receptor binding domains to 15 change the ultimate receptor binding characteristics of the chimeric heteromer.

The altered receptor binding domain may be taken from another TNF-like ligand receptor binding domain and grafted onto a polypeptide having a LT 20 association domain. Alternatively, a LT subunit association domain may be grafted onto a TNF-like ligand polypeptide having a TNF-like receptor binding domain. Receptor binding domains or LT subunit association domains may also be remodeled or re- 25 engineered by appropriate mutagenesis based on sequence and structural comparisons, and knowledge about which residues are critical for receptor binding and for subunit association, respectively.

The term "LT heteromeric (or heterotrimeric) 30 inhibitor" refers to a LT heteromeric complex, a modified LT heteromeric complex or a chimeric LT heteromeric complex which can specifically bind to a TNF or TNF-like receptor but which cannot induce multiple molecules of that receptor to aggregate on the 35 cell surface which normally triggers receptor signalling.

- 14 -

The term "ligand binding domain" refers to the portion or portions of a LT-, TNF- or TNF-like-receptor which is involved in specific recognition of and interaction with a LT, TNF or TNF-like ligand.

5       The term "monovalent ligand" refers to a ligand molecule (which may comprise multiple subunits) which can bind specifically to only a single receptor molecule with high affinity at one time, i.e., a ligand with only one functional receptor binding domain. In  
10 contrast, a "multivalent ligand" has more than one functional receptor binding domains and is thus capable of binding to and potentially clustering multiple receptor molecules.

15      The terms "receptor signalling" refers to all molecular reactions associated with the activation of a particular receptor pathway and subsequent molecular reactions which result therefrom.

20      A "type I leader sequence" is an amino-terminal portion of a eukaryotic protein which serves as a signal to direct the protein to the endoplasmic reticular (ER) membrane and often through the entire secretion pathway. The leader sequence is usually cleaved off by a signal peptidase in the ER membrane.

25      A "signal sequence" is the functional equivalent of a eukaryotic type I leader sequence in prokaryotic hosts, and directs the translocation of proteins into or across lipid bilayer membranes of a bacterium.

30      The term "mutation" refers to a change made in the specific sequence of amino acids of a polypeptide, or in the specific nucleic acid sequences which encode those amino acids. Mutations can alter a DNA, RNA or protein molecule at a single base or amino acid position, or at multiple adjacent or non-adjacent positions. Mutations can be transitions or transversions of a single base, or deletions,

- 15 -

insertions, inversions or translocations of nucleic acid sequences between regions of the same nucleic acid molecule or between different nucleic acid molecules.

Amino acid mutations can similarly be directed to a single position of the polypeptide chain, heterologous sequences can be inserted, or deletions or rearrangements of the amino acid sequence can be made.

The terms "receptor cross-linking" or "receptor clustering" refer to the physical aggregation of multiple adjacent receptor molecules in the plane of the cell surface membrane. Receptor clustering can act as a trigger, mediated by the intracellular domain(s) of the clustered and activated receptors, which starts a cascade of signalling events within the cell.

The terms "receptor binding domain" and "receptor binding loop" refer to the region or regions of a monomeric subunit comprising part of the receptor binding site of an oligomeric ligand containing that subunit, which contributes to or is capable of conferring receptor binding specificity. Residues critical for receptor binding are often found in a loop structure displayed from a central core as determined by three dimensional sequence modeling and on crystal structure analyses of various TNF family ligands.

The term "TNF-related ligand" refers to a ligand which may be classified as a member of the TNF family of ligands based on certain sequence conservation and on certain structural homologies which are conserved among family members.

The term "TNF (ligand) homology region" refers to amino acid residues located in the primary amino acid sequences of TNF family ligands which are conserved and which serve to identify TNF ligand family members. The conserved homology regions appear to contribute to the formation of a three dimensional "B-

- 16 -

sandwich" structure comprising two antiparallel  $\beta$ -pleated sheets.

**TNF and LT Subunits Trimerize into Multivalent Ligands**

Within the TNF family, even the most related  
5 ligands --TNF, LT- $\alpha$  and LT- $\beta$  -- are only about 50%  
homologous at the amino acid level. Localized sequence  
comparisons between the TNF ligand family reveal  
several regions of homology comprising conserved amino  
acid residues which serve as hallmark features for  
10 identifying TNF-related ligands.

TNF ligand homology regions are restricted to  
about 150 amino acid residues in the C-terminal portion  
of the proteins. These regions appear to form a  $\beta$ -  
sandwich structure from two anti-parallel  $\beta$ -pleated  
15 sheets (a classic viral "jelly-roll" motif; Jones et  
al., *Nature*, 338, pp. 225-28 (1989)). The  $\beta$ -sandwich  
is thus a structural motif common to the TNF ligands.

A variety of physical and chemical analyses  
of native and crystallized complexes reveal that both  
20 TNF and LT- $\alpha$  exist as homotrimers. The TNF and LT- $\alpha$   
wedge-shaped monomer subunits associate by packing  
about a three-fold axis of symmetry to form a compact  
conical trimer in which the face of one subunit  
contacts the edge of an adjacent subunit.

25 The TNF homology regions in TNF and LT- $\alpha$   
localize to the internal interfaces between adjacent  
interacting subunits of the trimeric ligand. The  
ability to trimerize is encoded in these residues which  
line the contact points between subunits. These  
30 internal residues define a scaffold structure upon  
which the external residues that contact a specific  
receptor are displayed within subunit clefts. These  
conserved regions which drive assembly of monomeric  
subunits into the trimeric "scaffold" of an active TNF  
35 ligand are referred to herein as "subunit association"

- 17 -

domains", and the sequences which comprise them called "subunit association sequences".

The most conserved amino acid regions among the other TNF ligands also fall within stretches of  $\beta$ -5 sandwich structures, and thus are likely to be analogous subunit association sequences. A sequence alignment of the extracellular portions of five TNF ligand family members is shown in **Figure 1**. Residues known to be involved in TNF and LT- $\alpha$  trimerization 10 (subunit association sequences) are underlined.

Structural models show that the CD40L sequence can form an energetically favorable scaffold  $\beta$ -sheet structure like TNF and LT- $\alpha$  subunits. It is expected that many TNF ligand family members, and 15 especially the more related TNF, LT- $\alpha$ , LT- $\beta$ , CD40L and FasL ligands can assume similar structures. Since the subunit conformations of the TNF-related ligands are likely to be similar, the trimeric structures which assemble from these subunits may also be conserved.

20 The subunits of most TNF ligands do not randomly form mixed multimers. Thus specific sequences within the conserved subunit association domains must guide  $\beta$ -sandwich structures to preferentially self-associate into a homotrimeric scaffold. LT- $\alpha$  and LT- $\beta$  25 subunits are exceptions. Each can form homotrimers and mixed heterotrimers. Presumably, the LT- $\alpha$  and LT- $\beta$  subunit association domains comprise specific residues which permit association between both heterologous and homologous subunits.

30 Intrinsic to the propensity for LT subunits to trimerize is their ability to form multivalent active ligands. One active form is a soluble trimer consisting of three LT- $\alpha$  subunits. The unique molecular environment of the cleft formed between 35 adjacent LT- $\alpha$  subunits in a LT- $\alpha$ 3 trimer accounts for TNF-R receptor binding specificity (Zhang et al., J.

- 18 -

Biol. Chem., 267, pp. 24069-75 (1992); Banner et al., Cell, 73, pp. 431-45 (1993). Another multivalent form (found in trace amounts in the membrane) consists of a trimer of two  $\alpha$  subunits and one  $\beta$  subunit (LT- $\alpha$ 2/ $\beta$ 1).

- 5 In a manner similar to receptor binding to LT- $\alpha$ 3, LT- $\beta$ -R binds the LT- $\alpha$ 2/ $\beta$ 1 ligand in the cleft formed between adjacent LT- $\alpha$  subunits, and with weaker affinity to the cleft formed between adjacent  $\alpha$ / $\beta$  subunits (Browning et al., J. Immunol., 154, pp. 33-46 (1995)). For the third  
10 trimer, the LT- $\alpha$ 1/ $\beta$ 2 (surface form) LT- $\beta$ -R binds to the  $\beta$ : $\beta$  cleft and weakly to a  $\beta$ : $\alpha$  and/or  $\alpha$ : $\beta$  cleft.

The multivalent nature of the TNF ligands is, in turn, responsible for mediating ligand-induced receptor activation by clustering multiple adjacent  
15 receptor molecules on the cell surface. For example, three, free TNF receptors can bind to LT- $\alpha$ 3. Each TNF receptor binds to the  $\alpha$ : $\alpha$  cleft between adjacent LT- $\alpha$  subunits. Receptor clustering triggers signalling by the intracellular portion of the clustered receptors,  
20 which can cascade into multiple signals involving downstream effector molecules that participate in subsequent events associated with the receptor signalling pathway.

The internal scaffold structure which forms  
25 from trimerized LT subunit association domains can be exploited in various ways to make heteromeric trimers that have only one receptor binding site (i.e. are monovalent ligands) for a particular receptor. A trimer with only one receptor binding site (e.g.  
30 a LT- $\alpha$ 2/ $\beta$ 1 heterotrimer in which the preferred receptor binding site lies in the one cleft between adjacent  $\alpha$ : $\alpha$  subunits) would inhibit the receptor clustering event that initiates signalling by that receptor or group of receptors, thereby inhibiting subsequent receptor  
35 signalling.

- 19 -

The present invention covers all forms of LT heteromers that complex between subunit association domain  $\beta$ -sandwich structures present in LT- $\alpha$  and LT- $\beta$  monomer subunits. Attached to this LT trimeric scaffold can be the original LT- $\alpha$  or LT- $\beta$  sequences (i.e., the parent molecules themselves) or sequences of either LT- $\alpha$  or LT- $\beta$  comprising mutations within receptor binding regions. These mutations can be made by substitution, deletion or insertion of amino acid sequences, and can be of three forms: those that convert heteromeric clefts into inactive clefts; those that alter but do not inactivate receptor binding in heteromeric clefts; and those that convert entire LT- $\alpha/\alpha$  or LT- $\beta/\beta$  clefts into new clefts that will bind a different receptor (see below).

In addition, LT subunit association sequences can be introduced into related TNF ligand subunit molecules to construct chimeric subunits which comprise a heterologous receptor binding domain and a LT subunit association domain. LT chimeric heterotrimers assembled from a wild type LT subunit and two LT chimeric subunits are essentially monovalent and can bind only one heterologous TNF-like receptor molecule with high affinity. The present invention also covers LT chimeric heterotrimers which can inhibit clustering and signalling by the corresponding heterologous receptor.

#### Production of Membrane-bound LT- $\alpha/\beta$ Complexes

Cell surface lymphotoxin complexes have been characterized in CD4 $^{+}$  T cell hybridoma cells (II-23.D7) that express high levels of LT (Browning et al., J. Immunol., 147, pp. 1230-37 (1991); Androlewicz et al., J. Biol. Chem., 267, pp. 2542-47 (1992)). Mature LT- $\alpha$  lacks a transmembrane domain and is localized to the cell surface through interaction with at least one

- 20 -

membrane-bound LT- $\beta$  subunit. Membrane-bound (surface) LT- $\alpha/\beta$  heteromeric complexes have predominantly a LT- $\alpha_1/\beta_2$  stoichiometry.

LT- $\beta$  as a cell membrane protein binds LT- $\alpha$  during synthesis, thus "targeting" the LT- $\alpha$  to the cell membrane. In the absence of LT- $\beta$ , LT- $\alpha$  is secreted into the extracellular medium. LT subunits normally assemble into complexes inside the cell prior to protein export into the membrane. Once LT- $\beta$  subunits are inserted into the membrane, they do not form stable complexes with secreted LT- $\alpha$ . Thus if the membrane-bound form of a LT- $\alpha/\beta$  heteromeric complex is desired, it is preferable to co-express the desired LT- $\alpha$  and LT- $\beta$  subunits within the same cell.

The surface LT- $\alpha/\beta$  heteromeric complex can be reconstructed by co-transfection of host cells with both the LT- $\alpha$  and LT- $\beta$  genes. Surface LT complexes cannot be observed on stable cell lines which express either LT gene alone. However, if the host cell normally produces large amounts of LT- $\alpha$  (e.g. RPMI 1788 cells; see below), then transfection with a LT- $\beta$  gene which encodes the desired LT- $\beta$  polypeptide should be sufficient to generate LT- $\alpha/\beta$  complexes comprising non-recombinant LT- $\alpha$  subunits.

Co-expression of LT- $\alpha$  and LT- $\beta$  polypeptides in a number of eukaryotic expression systems leads to their assembly and export as active ligand (Crowe et al., *J. Immunol. Methods*, 168, 79-89 (1994)). Host systems that can be used include but are not limited to CHO cells, COS cells, B cells including myelomas, baculovirus-infected insect cells and yeast.

The LT- $\alpha$  subunit of the LT heteromeric complexes of this invention can be selected from lymphotoxin- $\alpha$ , native human or animal lymphotoxin- $\alpha$ , recombinant lymphotoxin- $\alpha$ , soluble lymphotoxin- $\alpha$ , secreted lymphotoxin- $\alpha$ , lymphotoxin- $\alpha$  muteins having

- 21 -

LT- $\alpha$  biological activity, or lymphotoxin- $\alpha$  fragments of any of the above having LT- $\alpha$  biological activity.

The LT- $\alpha$  polypeptide of the LT heteromeric complexes of this invention can be any soluble form of 5 the molecule including active fragments thereof which can be produced in eukaryotic expression systems, wherein the natural LT- $\alpha$  leader sequence will be cleaved off. Alternatively, fusions of the mature LT- $\alpha$  sequence with a heterologous signal sequence can be 10 used to maximize the secretion of LT- $\alpha/\beta$  heteromeric complexes in other host systems. Signals are chosen based on the intended host cell, and may include bacterial, yeast, mammalian and viral sequences. The native signal, or the vascular cell adhesion molecule-1 15 (VCAM-1) signal sequence, is suitable for use in mammalian expression systems.

The LT- $\alpha$  polypeptides of the LT heteromeric complexes of this invention can also be fused to polypeptides having a prolonged plasma half-life such 20 as immunoglobulin chains or fragments thereof. Plasma proteins which may be used to enhance plasma half-life include serum albumin, immunoglobulins, apolipo- proteins, and transferrin. Polyethylene glycol (PEG) attachment may stabilize the polypeptide and lower its 25 immunogenicity. Preferably the LT- $\alpha$  fusion protein is not significantly immunogenic in the subject to be treated and the plasma protein does not cause undesirable side effects in subjects due to its normal biological activity.

30 Human LT- $\alpha$  is glycosylated on N and O residues, and depending on the source, exhibits considerable sugar-based microheterogeneity. The oligosaccharide composition of the particular LT- $\alpha$  chosen to form the LT complex may affect in vivo 35 clearance rates (Fukushima et al., Arch. Biochem. Biophys., 304, pp. 144-53 (1993)). Since glycosylation

- 22 -

variants can be produced by expression in different host cells, this is one factor to be considered in selecting a source of LT- $\alpha$  for the LT heteromeric complexes of this invention.

- 5       LT- $\alpha$  can be purified from a B lymphoblastoid line RPMI 1788, which constitutively secretes LT- $\alpha$  and which can be induced to secrete higher levels by treating with the phorbol ester PMA (Aggarwal et al., J. Biol. Chem., 259, pp. 686-91 (1984)).
- 10      Alternatively, the cloned human LT- $\alpha$  gene can be used to recombinantly produce the LT- $\alpha$  polypeptides of the LT heteromeric complexes of this invention in different host systems including bacteria (Schoenfeld et al., J. Biol. Chem., 266, pp. 3863-69 (1991)); baculovirus-
- 15      infected insect cells (Crowe et al., J. Immunol. Methods, 168, pp. 70-89 (1994)); and mammalian cells (Browning and Ribolini, J. Immunol., 143, pp. 1859-67 (1989); Fukushima et al., Arch. Biochem. Biophys., 304, pp. 144-53 (1993)).
- 20      Portions of the LT- $\alpha$  gene which encode polypeptide fragments having LT- $\alpha$  biological activity can be evaluated using routine screening assays. Useful screening assays for LT- $\alpha$  biological activity include competitive inhibition assays with native LT- $\alpha$
- 25      bound to TNF-R, or measuring either directly or indirectly by inhibition the ability of the LT- $\alpha$  to induce cytotoxicity of tumor cells in assays known to the art. Preferably, LT- $\alpha$  fragments are assembled into heteromeric complexes with LT- $\beta$  and the complexes
- 30      assayed for LT- $\alpha/\beta$  biological activity by competitive inhibition with LT- $\alpha/\beta$  bound to LT- $\beta$ -R, or for their ability to induce cytotoxicity of tumor cells in the assays disclosed herein.

35      Lymphotoxin- $\beta$ , also referred to as p33, has been identified on the surface of T lymphocytes, T cell lines, B cell lines and lymphokine-activated killer

- 23 -

cells. LT- $\beta$  is the subject of applicants' co-pending international applications PCT/US91/04588, published January 9, 1992 as WO 92/00329; and PCT/US93/11669, published June 23, 1994 as WO 94/13808, which are 5 herein incorporated by reference.

The LT- $\beta$  gene encodes a polypeptide of 240-244 amino acids (Browning et al., *Cell*, 72, pp. 847-56 (1993)). LT- $\beta$  is a type II membrane protein with a short N-terminal cytoplasmic domain followed by a 10 membrane anchoring domain of 30 hydrophobic amino acids. It has a single N-linked glycosylation site and has only one cysteine residue which does not appear to be involved in intersubunit disulfide bond formation.

The LT- $\beta$  subunits comprising the LT 15 heteromeric complexes of the present invention can be selected from lymphotoxin- $\beta$ , native human or animal lymphotoxin- $\beta$ , recombinant lymphotoxin- $\beta$ , soluble lymphotoxin- $\beta$ , secreted lymphotoxin- $\beta$ , lymphotoxin- $\beta$  muteins having LT- $\beta$  biological activity, or 20 lymphotoxin- $\beta$  fragments of any of the above having LT- $\beta$  biological activity.

As discussed above for the LT- $\alpha$  polypeptide, the LT- $\beta$  polypeptides can also be modified to increase their solubility or plasma half-life using the same 25 methods. Likewise, portions of the LT- $\beta$  gene which encode polypeptide fragments having LT- $\beta$  biological activity can be evaluated using routine screening assays as discussed for LT- $\alpha$ .

#### **Production of Soluble LT Heteromeric Complexes**

This invention is based on the ability of the 30 LT- $\alpha$  and LT- $\beta$  proteins to form either LT- $\alpha$ 1/ $\beta$ 2 or LT- $\alpha$ 2/ $\beta$ 1 heterotrimers. LT- $\alpha$  is normally a secreted protein, but is sequestered at the cell surface by binding to surface LT- $\beta$ . By removal of the LT- $\beta$  35 transmembrane region and replacement with a leader

- 24 -

sequence targeting that LT- $\beta$  through the secretion pathway, LT- $\alpha/\beta$  trimers can be prepared in a soluble form.

Soluble (non-membrane-bound) LT- $\alpha/\beta$

- 5 heteromeric complexes comprise LT- $\beta$  subunits which have been changed from a membrane-bound to a soluble form. These complexes are described in detail in applicants' co-pending international application (PCT/US93/11669, published January 9, 1992 as WO 94/13808). Soluble  
10 LT- $\beta$  peptides are defined by the amino acid sequence of lymphotoxin- $\beta$  wherein the sequence is cleaved at any point between the end of the transmembrane region (i.e. at about amino acid #44) and the first TNF homology region (i.e. at amino acid #88) according to the  
15 numbering system of Browning et al., Cell, 72, pp. 847-56 (1993).

Soluble LT- $\beta$  polypeptides may be produced by truncating the N-terminus of LT- $\beta$  to remove the cytoplasmic tail and transmembrane region (Crowe et  
20 al., Science, 264, pp. 707-710 (1994)). Alternatively, the transmembrane domain may be inactivated by deletion, or by substitution of the normally hydrophobic amino acid residues which comprise a transmembrane domain with hydrophilic ones. In either  
25 case, a substantially hydrophilic hydropathy profile is created which will reduce lipid affinity and improve aqueous solubility. Deletion of the transmembrane domain is preferred over substitution with hydrophilic amino acid residues because it avoids introducing  
30 potentially immunogenic epitopes.

The deleted or inactivated transmembrane domain may be replaced with or attached to a type I leader sequence (e.g. the VCAM-1 leader) such that the protein is secreted beginning with a sequence anywhere  
35 from between val40 to pro88. Soluble LT- $\beta$  polypeptides may include any number of well-known leader sequences

- 25 -

at the N-terminus. Such a sequence would allow the peptides to be expressed and targeted to the secretion pathway in a eukaryotic system. See, e.g., Ernst et al., United States Patent No. 5,082,783 (1992).

5 Soluble LT- $\alpha/\beta$  heteromeric complexes may be produced by co-transfected a suitable host cell with DNA encoding LT- $\alpha$  and soluble LT- $\beta$  (Crowe et al., J. Immunol. Methods, 168, pp. 79-89 (1994)). Soluble LT- $\beta$  secreted in the absence of LT- $\alpha$  is highly  
10 oligomerized. However, when co-expressed with LT- $\alpha$ , a 70 kDa trimeric-like structure is formed which contains both proteins. It is also possible to produce soluble LT- $\alpha_1/\beta_2$  heteromeric complexes by transfecting a cell line which normally expresses only LT- $\alpha$  (such as the  
15 RPMI 1788 cells discussed above) with a gene encoding a soluble LT- $\beta$  polypeptide.

LT- $\alpha$  and LT- $\beta$  polypeptides may be separately synthesized, denatured using mild detergents, mixed together and renatured by removing the detergent to  
20 form mixed LT heteromeric complexes which can be separated (see below).

#### Purification of LT- $\alpha_2/\beta_1$ Complexes

The ability of various LT- $\alpha/\beta$  heteromers to bind to different receptors can be exploited as a  
25 method for purifying a specific LT heteromeric form from a mixture of heteromers of differing compositions. Homogeneous receptors are immobilized onto a matrix and preferably, are packed into a column. A mixture containing heteromeric ligands is then mixed with, or  
30 passed over a column of the affinity matrix under conditions wherein the ligands bind to the matrix-linked receptors. Heteromeric ligands of a specific composition can then be purified by differentially eluting ligands with lower receptor binding constants  
35 with a gradual or step-wise change in the conditions

- 26 -

that affect ligand-receptor interaction (i.e., salt and pH). The use of either a single affinity column or combinations of affinity columns to purify specific LT heteromeric molecules is envisioned by this invention.

5 Soluble LT- $\alpha$ 2/ $\beta$ 1 heteromeric complexes are separated from co-expressed complexes comprising a different subunit stoichiometry by chromatography using TNF and LT- $\beta$  receptors as affinity purification reagents. The TNF receptors only bind within  $\alpha/\alpha$  10 clefts of LT complexes. The LT- $\beta$  receptor binds with high affinity to  $\beta/\beta$  clefts, and with lower affinity to  $\alpha/\beta$  clefts of heteromeric LT- $\alpha/\beta$  complexes.

Accordingly, LT- $\alpha$ 3 and LT- $\alpha$ 2/ $\beta$ 1 will bind to TNF-R. The LT- $\beta$ -R can also bind LT- $\alpha$ 2/ $\beta$ 1 trimers 15 (within the  $\alpha/\beta$  clefts) but cannot bind LT- $\alpha$ 3. In addition, the LT- $\beta$ -R (but not TNF-R) binds LT- $\alpha$ 1/ $\beta$ 2 and LT- $\beta$ n (the exact composition of such preparation is unknown, however, they are large aggregates).

The receptor affinity reagents can be 20 prepared as either a soluble extracellular domain (see for example Loetscher et al., J. Biol. Chem., 266, pp. 18324-29 (1991)), or as chimeric proteins with the extracellular ligand binding domain coupled to an immunoglobulin Fc domain (Loetscher et al., J. Biol. Chem., 266, pp. 18324-29 (1991); Crowe et al., Science, 264, pp. 707-710 (1994)). Receptors are coupled to 25 affinity matrices by chemical cross-linking using routine procedures.

There are two schemes by which the LT- $\alpha$ 2/ $\beta$ 1 30 ligand can be purified (**Figure 2**). In the first scheme, a supernatant from an appropriate expression system co-expressing both LT- $\alpha$  and the truncated LT- $\beta$  form is passed over a TNF-R column. The bound LT- $\alpha$ 3 and LT- $\alpha$ 2/ $\beta$ 1 trimers are eluted via classical methods 35 such as chaotrophe or pH change. The eluate solution is neutralized or the chaotrophe removed, and the

- 27 -

solution is then applied to a LT- $\beta$ -R column. Only the LT- $\alpha$ 2/ $\beta$ 1 binds to the LT- $\beta$ -R and the LT- $\alpha$ 3 flows through the column. Elution of bound material yields purified LT- $\alpha$ 2/ $\beta$ 1 (Example 2).

5 In the second scheme, all LT- $\beta$ -containing forms (LT- $\beta$ (n), LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1) are bound to and eluted from a LT- $\beta$ -R column (LT- $\alpha$ 3 flows through this column). The LT- $\beta$ -containing forms are then passed over a TNF-R column, which binds only to the LT- $\alpha$ 2/ $\beta$ 1 trimer. LT- $\alpha$ 2/ $\beta$ 1 can then be eluted in a pure state. Both procedures yield the same preparation of LT- $\alpha$ 2/ $\beta$ 1.

10 15 In both cases, purified LT- $\alpha$ 2/ $\beta$ 1 trimers can be separated from LT- $\beta$  by subsequent gel filtration and/or ion exchange chromatographic procedures known to the art. Various combinations of affinity columns which can be used are diagrammed in Figure 2.

#### Purification of LT- $\alpha$ 1/ $\beta$ 2 Complexes

Soluble LT- $\alpha$ 1/ $\beta$ 2 heteromeric complexes are 20 separated from co-expression complexes comprising a different subunit stoichiometry by chromatography using TNF and LT- $\beta$  receptors as selective affinity purification reagents, as described above.

25 There are two schemes by which the LT- $\alpha$ 1/ $\beta$ 2 ligand can be purified using receptors and immuno-affinity chromatography. In the first scheme, a supernatant from an appropriate expression system co-expressing both LT- $\alpha$  and the truncated LT- $\beta$  form is passed over a TNF-R column. The TNF-R will bind LT- $\alpha$ 3 and LT- $\alpha$ 2/ $\beta$ 1 trimers. The flow through from the TNF-R column will contain LT- $\beta$ (n) and LT- $\alpha$ 1/ $\beta$ 2 (Example 3).

30 35 In the second scheme, all LT- $\beta$ -containing forms (LT- $\beta$ (n), LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1) are bound to and eluted from a LT- $\beta$ -R column using classical methods such as chaotropic or pH change. (LT- $\alpha$ 3 flows through

- 28 -

this column). The eluate is neutralized or the chaotropic removed, and the eluate is then passed over a TNF-R column, which binds only to the LT- $\alpha$ 2/ $\beta$ 1 trimers. The flow through of this column will contain  
5 LT- $\beta$ (n) and LT- $\alpha$ 1/ $\beta$ 2 trimers.

In both cases, purified LT- $\alpha$ 1/ $\beta$ 2 trimers can be separated from LT- $\beta$  by subsequent gel filtration and/or ion exchange chromatographic procedures known to the art. Various combinations of affinity columns  
10 which can be used are diagrammed in **Figure 2**.

#### Purification of Mutant and Chimeric LT Heteromers

The receptor purification schemes outlined above can be modified to purify variously altered forms of the LT heteromeric complexes by coupling a different  
15 receptor to a column matrix. For example, if the  $\beta$ / $\beta$  cleft of LT- $\alpha$ 1/ $\beta$ 2 is converted to a CD40R binding cleft by mutagenesis of residues involved in receptor binding, a CD40 receptor column could be used as a step to purify the chimeric LT/CD40 heterotrimers. A CD40  
20 receptor column could also be used to purify chimeric LT/CD40 heteromeric complexes assembled from CD40 subunits mutated in their subunit association domains to induce trimerization with LT- $\alpha$  or LT- $\beta$  subunits. In all cases, the various heteromeric trimers (i.e.,  
25 LT- $\alpha$ 1/ $\beta$ 2, LT- $\alpha$ 2/ $\beta$ 1 and mutant and chimeric forms thereof) can be resolved further by ion exchange chromatography, especially useful in the event that one of the columns described above no longer separates forms with altered binding characteristics.

30 Alternatively, different mutant and chimeric forms of LT- $\alpha$ / $\beta$  heteromeric complexes can be separated and purified by a variety of conventional chromatographic means well known to those of skill in the art. It may also be preferable to combine a series

- 29 -

of conventional purification schemes with one or more of the immunoaffinity purification steps described above.

**Physical Characterization of Purified LT Heterotrimers**

- 5 Any of a number of conventional methods can be used to characterize the subunit stoichiometry and relative purity of LT heteromeric complexes in fractions separated by immunoaffinity chromatography or other purification techniques as described above.
- 10 These include SDS polyacrylamide gel electrophoresis (SDS PAGE), gel exclusion chromatography, ion exchange chromatography and high pressure liquid chromatography (HPLC), all of which are well known methods of protein separation.
- 15 LT and other ligand subunits separated by gel electrophoresis may be detected by radiolabelling the proteins prior to electrophoresis, or by staining the gel after electrophoresis with dyes that bind polypeptides (such as coomassie blue) and performing 20 densitometry tracing. The proteins in the gel may also be blotted onto a membrane and probed. The probes may be enzymatically, fluorescently or radioactively-labelled anti-subunit antibodies, or another antibody which recognizes an immunological tag (such as the myc 25 epitope) incorporated into one or more of the ligand subunits ("Western blot"). All techniques are well known to the art.

Using these techniques, the LT- $\alpha/\beta$  heteromeric complexes purified according to the methods 30 set forth in Examples 2 and 3 using the combination of immunoaffinity columns as in **Figure 2** (right side) were characterized (Example 4) by SDS PAGE analysis. The purified LT- $\alpha/\beta$  heterotrimers were also individually analyzed by C4 reverse phase HPLC, and the relative 35 ratios of LT- $\alpha$  and LT- $\beta$  in each fraction is shown in

- 30 -

**Figure 3.** These experiments are consistent with the identification of the LT heteromeric complexes as heterotrimers having the subunit stoichiometries of LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1.

5 Gel exclusion sizing analysis of the purified LT- $\alpha$ / $\beta$  heteromeric complexes, using a TSK 3000 HPLC resin in a phosphate buffered saline buffer (**Figure 4a**, Example 4) confirmed that the LT heteromeric complexes behave as trimers of about 50-60kDa molecular weight.

10 Ion exchange chromatographic analyses of the purified LT heteromeric complexes (**Figure 4b**; Example 4) further confirmed the results of the experiments described above, and indicate that cross-contamination between different LT heteromeric forms is  
15 no greater than 1-5% after affinity purification. Taken together, these experiments identify the LT heteromeric complexes made according to the purification procedures described in Examples 2 and 3 as LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1 heterotrimers.

20 **Assessing LT Heteromeric Ligand-Receptor Binding**

The LT heteromeric complexes of this invention are capable of inhibiting receptor signalling by binding to a cell surface receptor molecule without mediating clustering of adjacent receptors. To qualify  
25 as an effective inhibitor, the LT heteromeric complexes must bind to the selected receptor with an affinity high enough to compete with endogenous multivalent ligands for binding sites on the receptor.

To determine whether a LT heteromeric complex  
30 can bind to a selected receptor, and whether it can compete with natural ligands of that receptor, ligand-receptor binding assays are performed.

The strength of the binding between a receptor and ligand can be measured using an enzyme-linked immunoabsorption assay (ELISA), a radio-

- 31 -

immunoassay (RIA), or other such immunodetection methods which are techniques well known in the art.

The ligand-receptor binding interaction may also be measured with the Biacore™ instrument which  
5 exploits plasmon resonance detection (Zhou et al., Biochemistry, 32, pp. 8193-98 (1993); Faegerstram and O'Shannessy, "Surface plasmon resonance detection in affinity technologies". In Handbook of Affinity Chromatography, pp. 229-52, Marcel Dekker, Inc., New  
10 York (1993)).

The Biacore™ technology allows one to bind receptor to a gold surface and to flow ligand over it. Plasmon resonance detection gives direct quantitation of the amount of mass bound to the surface in real  
15 time. This technique yields both on and off rate constants and thus a ligand-receptor dissociation constant and affinity constant can be directly determined.

With any of these or other techniques for  
20 measuring receptor-ligand interactions, one can evaluate the quality of a LT heteromeric inhibitor made from wild type, mutated, altered or chimeric LT and TNF-related ligand subunits.

The Biacore™ technique was used to generate a  
25 binding profile of LT- $\alpha$ 3, LT- $\alpha$ 2/ $\beta$ 1 and LT- $\alpha$ 2/ $\beta$ 1 heterotrimers to the LT- $\beta$ -R and p55 TNF-R (Example 5). **Figure 5a** characterizes the binding profiles of the various LT heterotrimer forms to p55 TNF-R and to LT- $\beta$ -R (**Figure 5b**) as a function of time. **Figure 5a**  
30 shows that LT- $\alpha$ 3 and LT- $\alpha$ 2/ $\beta$ 1 -- but not LT- $\alpha$ 1/ $\beta$ 2 -- bind specifically to TNF-R. **Figure 5b** shows that both LT- $\alpha$ 2/ $\beta$ 1 and LT- $\alpha$ 1/ $\beta$ 2 bind LT- $\beta$ -R, but that LT- $\alpha$ 2/ $\beta$ 1 heterotrimers bind with a lower affinity.

- 32 -

**LT- $\alpha/\beta$  Heterotrimers Can Inhibit Receptor Signalling**

Once it is established that a LT heteromeric complex can bind to a selected target receptor and preferably, that it can compete with endogenous ligands 5 for receptor binding sites, an assay to monitor the activity of that selected receptor once it is bound to the LT heteromeric ligand must be performed. Assays known to be reliable indicators of signalling by the selected receptor will, of course, be preferred. In 10 any event, at least one activity associated with the activation of the selected receptor should be measured to assess the ability of the potential LT heteromeric inhibitor, when bound to the selected receptor, to inhibit receptor signalling.

15 Assays which monitor the activity of related but non-selected receptors by the addition of a putative LT heteromeric inhibitor can also be performed. Such assays are useful in detecting co-activated or co-inhibited receptors which are 20 stimulated or inhibited by the LT heteromeric inhibitor preparation. A stimulatory or inhibitory effect on the non-selected receptor might be due to cross-reaction of the purified LT heteromeric ligand with the non-selected receptor. Alternatively, these effects might 25 be due to low levels of a contaminating LT heteromeric form(s) in the purified preparation which can activate or inhibit the non-selected receptor.

The soluble LT- $\alpha_2/\beta_1$  heterotrimer is claimed herein as an inhibitor of the p55 TNF-R and p75 TNF-R. 30 LT- $\alpha_2/\beta_1$  can bind within its one  $\alpha/\alpha$  cleft to a TNF receptor, but not within its two  $\alpha/\beta$  clefts. Because this LT- $\alpha_2/\beta_1$  heterotrimer is monovalent for TNF-R, it cannot cluster or "cross-link" multiple surface TNF-R molecules and thus it blocks normal TNF-R signalling 35 activity.

- 33 -

Purified LT- $\alpha$ 2/ $\beta$ 1 can inhibit the action of TNF or LT- $\alpha$  in a number of culture systems. For example, the mouse L929 cell can be killed by TNF or LT- $\alpha$  in either short term cytolytic assays or in longer term growth inhibition assays (Example 6). The short term cytolytic assay actually constitutes the standard analytical tool for assessing the biological activity of these two cytokines. These assays have been previously described (Browning et al., J. Immunol., 10 143, pp. 1859-67 (1989)).

**Figure 6a** shows that LT- $\alpha$ 2/ $\beta$ 1 can block TNF action in mouse L929 cytolytic assays and in related growth inhibition assays using the human tumor lines Me180 (**Figure 6b**) and HT29 (**Figure 6c**). In assays using Me180 and HT29 tumor cell lines, interferon- $\alpha$  is included since it acts synergistically with TNF to inhibit cell growth. The LT- $\alpha$ 1/ $\beta$ 2 form, which cannot bind to the p55 TNF-R, is inactive in blocking TNF activity (not shown).

These experiments show that purified LT- $\alpha$ 2/ $\beta$ 1 can compete with natural TNF for binding to TNF-R, thereby inhibiting TNF-R signalling activity. These assays are considered representative of physiological TNF activity.

In other studies, LT- $\alpha$ 2/ $\beta$ 1 was able to block the TNF-induced increase in VCAM and ELAM on human umbilical vein endothelial cell surfaces, which is a reliable assay for the inflammatory activity of TNF (P. Vassalli, Annu. Rev. Immunol., 10, pp. 441-52 (1992)).

In **Figure 7**, the ability of a soluble LT- $\beta$ -R ( $\text{LT-}\beta\text{-R-Fc}$ ) to bind to LT molecules displayed on the surface of a T cell hybridoma is used as a model for assaying surface LT signalling to another receptor-positive cell (Example 7). This experiment shows that purified LT- $\alpha$ 1/ $\beta$ 2 can block the binding of soluble LT- $\beta$ -Rs to a T cell hybridoma displaying surface

- 34 -

LT- $\alpha/\beta$  complexes. This result demonstrates that the soluble LT- $\alpha_1/\beta_2$  molecules of this invention can effectively compete with natural cell surface LT molecules for binding to the LT-R. Its ability to 5 compete with cell surface LT in this assay supports the notion that soluble LT- $\alpha_1/\beta_2$ , or mutated or altered forms thereof (see below), will be useful as a therapeutic agent to inhibit signalling by the LT-R.

10 **Mutagenesis to Refine the Binding Specificity of LT Heteromeric Inhibitors**

LT heteromeric inhibitors (e.g. LT- $\alpha_2/\beta_1$ ) may exhibit cross-reactivity or background binding activity to other known or unknown receptors. This can interfere with the desired result of inhibiting certain 15 signalling pathways. Thus, certain LT- $\alpha/\beta$  heteromers may mediate non-productive monovalent interactions or may abnormally bridge different receptor molecules resulting in "confused receptor aggregation". This interaction with additional receptors may be reduced or 20 eliminated by mutagenesis of exposed amino acid residues of an LT subunit which lie within a receptor binding cleft not involved in binding to and inhibiting the selected receptor. For example, the LT- $\beta$  subunit may be mutagenized to eliminate receptor binding 25 activity in one of the LT- $\alpha/\beta$  clefts of a LT- $\alpha_2/\beta_1$  heterotrimeric inhibitor. Mutations in either LT- $\alpha$  or LT- $\beta$  subunits in the soluble LT- $\alpha_1/\beta_2$  or LT- $\alpha_2/\beta_1$  complexes could increase the specificity of inhibitors assembled from them.

30 Similarly, mutagenesis of exposed LT subunit residues can also be useful in eliminating residual receptor binding activity due to low levels of alternate trimeric forms contaminating a purified LT heterotrimer preparation. For example, LT- $\alpha_1/\beta_2$  35 preparations typically exhibited some trace cytotoxic

- 35 -

activity when assayed using the WEHI 164 or L929 cytolytic assays (Example 6). This activity could be blocked by anti-LT- $\alpha$  monoclonal antibodies (mAbs) or by soluble p55 TNF-R, suggesting that it was caused by

5 residual levels of LT- $\alpha$ 3 trimeric forms in the LT- $\alpha$ 1/ $\beta$ 2 trimer preparations. In addition, it might be useful to eliminate LT- $\alpha$  binding to TNF-R to enhance the selectivity of a particular LT heterotrimer for another receptor.

10 To reduce or eliminate the binding of the soluble heteromeric trimers to a receptor, either directed or random mutagenesis using available techniques can be performed on amino acid residues predicted to be exposed within the cleft between two 15 subunits. Mutagenized proteins are then assembled into trimers either by expressing and isolating individual subunits and mixing them *in vitro*, or by co-expression of subunit genes *in vivo*. Heteromeric ligands are purified according to procedures described in Examples 20 2 and 3, and the purified forms functionally screened and selected based on altered receptor binding characteristics using standard immunological techniques and the procedures set forth in Examples 5-7.

For example, LT- $\alpha$ 2/ $\beta$ 1 can bind to and inhibit 25 aggregation of TNF-R, as shown above. In addition, LT- $\alpha$ 2/ $\beta$ 1 heterotrimers also bind LT- $\beta$ -R within the  $\alpha/\beta$  clefts (Figure 5b), and thus can also compete for binding between LT- $\beta$ -R and cell surface LT molecules in the II-23 cell assay described above.

30 LT- $\alpha$ 2/ $\beta$ 1 binds to LT- $\beta$ -R with lower affinity than to p55 TNF-R. To target LT- $\alpha$ 2/ $\beta$ 1 binding exclusively to LT- $\beta$ -R, p55 TNF-R binding can be reduced or eliminated by mutagenizing specific residues within the  $\alpha/\alpha$  cleft. Modified LT- $\alpha$ 2/ $\beta$ 1 heterotrimers comprising mutant LT- $\alpha$  35 subunits that no longer bind TNF-R would act as selective inhibitors of the LT- $\beta$ -R signalling pathway.

- 36 -

Mutations have been described in LT- $\alpha$  that eliminate the ability of LT- $\alpha$ 3 to trigger cell death in the mouse WEHI 164 line. One example is the "D50N mutation" which changes Asp50 to Asn50 and eliminates 5 LT- $\alpha$ 3 binding to TNF-R (Goh and Porter, Protein Eng., 4, p. 385 (1991); Van et al., Protein Eng., 7, p. 5 (1994)).

The D50N mutation was introduced into the LT- $\alpha$  subunits of the various LT- $\alpha$ / $\beta$  heteromeric forms 10 and the modified ligands tested in receptor binding and ligand competition assays (Example 8). **Figure 8a** shows the growth inhibition activity of wild-type LT- $\alpha$ 1/ $\beta$ 2 and mutant LT- $\alpha$ (D50N)1/ $\beta$ 2 trimers on WEHI 164 cells. Normal preparations of LT- $\alpha$ 3 trimers show 50% 15 growth inhibition at 1-10 pg/ml, and wild-type LT- $\alpha$ 1/ $\beta$ 2 typically retain 0.1-1% LT- $\alpha$ -like activity. The D50N mutation, which abolishes activity of normal LT- $\alpha$ 3 on these cells, also eliminated the residual LT- $\alpha$ -like activity in the altered LT- $\alpha$ 1/ $\beta$ 2 preparation.

20 **Figure 8b** shows that the LT- $\alpha$ (D50N)1/ $\beta$ 2 modified heteromeric complex retains full specific activity in a WiDr cytotoxicity assay which measures signalling through the LT- $\beta$ -R (i.e., an antibody directed against LT- $\beta$ -R will block this activity). 25 These experiments show that a mutation can be made in the LT- $\alpha$  subunit of a LT heterotrimer that eliminates residual TNF-R signalling but does not affect the ability of the modified heterotrimer to signal through the LT- $\beta$ -R.

30 Mutations can also be introduced in LT subunits to influence the relative binding affinity of a LT heteromer for one receptor type over another. For example, single amino acid replacements have been made in the TNF ligand that limit its binding to either p55 35 TNF-R or p75 TNF-R but not both receptors (Van et al.,

- 37 -

Nature, 361, pp.266-69 (1993)). It is likely that similar point mutations can be engineered into the LT- $\alpha$  subunit gene to restrict the binding of a LT heteromer comprising that modified LT- $\alpha$  subunit to one of the two TNF receptors. In this case, a modified LT- $\alpha$ 2/ $\beta$ 1 heterotrimer with the restricted LT- $\alpha$  form would be a selective inhibitor of only one receptor form. As these receptors mediate different signals, the modified LT- $\alpha$ 2/ $\beta$ 1 heterotrimer would be more selective in its inhibitory properties, and would likely be more useful clinically.

Similarly, the TNF-R binding domain could be grafted onto the LT- $\alpha$  (or the LT- $\beta$ ) subunit association domain (described below), and such point mutations that specifically destroy one or the other form of TNF-R could be incorporated into these modified LT subunits. Chimeric TNF/LT heterotrimers assembled from the modified LT subunits would be selective inhibitors of only one form of TNF-R.

Soluble LT- $\alpha$ 1/ $\beta$ 2 heterotrimers bind to the LT- $\beta$ -R and induce signalling. The exact nature of which receptors must be aggregated to initiate LT- $\beta$ -R signalling is currently unclear. Nonetheless, each cleft in the LT- $\alpha$ 1/ $\beta$ 2 trimer is unique and is likely to bind only one receptor type with high affinity. Therefore, the LT- $\alpha$ 1/ $\beta$ 2 trimer can also be used as an inhibitor of LT- $\beta$ -R signalling. Mutations in the LT- $\alpha$  subunit of the trimer may be required to eliminate binding of other as yet unidentified receptors to LT- $\alpha$ /LT- $\beta$  clefts. As these interactions are likely to involve binding modes similar to those involved in p55 TNF-R, mutations in LT- $\alpha$  that are known to eliminate p55 TNF-R binding are reasonable candidates.

It may be desirable to make similar changes to block the ability of LT- $\beta$  to bind to LT- $\beta$ -R as discussed above. There are examples in the art of

- 38 -

- mutagenizing specific residues in TNF, LT- $\alpha$  and CD40L that eliminate receptor binding (see e.g., Goh and Porter, Protein Eng., 4, pp. 385, 785 (1991); Van et al., Nature, 361, pp. 266-69, see comments (1993)). By 5 comparing the TNF and CD40L data, one can predict which residues, if changed, would eliminate binding of a LT heteromeric ligand to LT- $\beta$ -R. Such residues can be made using conventional mutagenesis approaches and tested using the procedures described herein. By 10 incorporating such mutations into the LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1 heterotrimers, one could enhance their selectivity and reduce any undesirable side effects caused by background levels of promiscuous receptor binding.
- 15 One could create a form of LT- $\alpha$ 1/ $\beta$ 2 that could inhibit LT- $\alpha$ 1/ $\beta$ 2 function (e.g. LT- $\beta$ -R activation) by mutagenizing the LT- $\alpha$  subunit to eliminate other signalling components. In practice, one would begin by introducing the mutations in LT- $\alpha$  20 that are already known to affect LT- $\alpha$  activity and then ask whether a LT- $\alpha$ 1/ $\beta$ 2 molecule assembled with such an LT- $\alpha$  mutation retained activity using cytotoxicity or growth inhibition assays such as those described in Example 6. Mutations that eliminated activity while 25 retaining LT- $\beta$ -R binding (Example 5) would be chosen as putative therapeutic LT heteromeric inhibitors. The ability to specifically modulate receptor binding enhances the utility of LT- $\alpha$ / $\beta$  heterotrimers as therapeutic agents.
- 30 **Altering the Subunit Association Domains of TNF-Related Ligand Subunits to Make Chimeric LT Heteromeric Inhibitors**

This invention also relates to the use of sequences of LT- $\alpha$  and LT- $\beta$  which, when inserted in the 35 appropriate context, allow the molecule to associate with similar sequences of another LT subunit. Such

- 39 -

association sequences, when spaced with amino acids stretches of an appropriate length, fold into a TNF-like structure capable of associating with other TNF-like structures to form heteromeric trimers. The 5 TNF-like association sequences behave as a structural and functional domain, and can thus be inserted as a separable unit into a molecule to enable it to trimerize. The subsequent trimer represents a unique structural scaffold onto which new domains of sequence 10 can be built. The structure of the  $\alpha$ -carbon backbone of LT- $\alpha$  with the residues critical for trimer formation highlighted is shown in **Figure 9**.

**CD40/LT Chimeric Inhibitors.** A heteromeric molecule capable of binding to the CD40 receptor 15 (CD40R) may consist of one LT- $\beta$  and two CD40L/LT- $\alpha$  chimeric subunits. The chimeric subunits should possess sufficient portions of the internal scaffold region to allow association of the CD40L/LT- $\alpha$  chimeric subunits with LT- $\beta$ , and should also retain as much 20 CD40L sequence as possible to retain maximal CD40 receptor binding cleft sequences and to minimize the amount of potentially antigenic chimeric sequences formed. Thus, a CD40L-LT chimera may consist of either the "association sequences" of LT- $\alpha$  engrafted onto the 25 CD40L structure or the ligand binding domain of CD40L engrafted onto the LT- $\alpha$  core association sequences. Conversely, a heteromeric molecule capable of binding to CD40R may consist of one LT- $\alpha$  and two CD40L/LT- $\beta$  chimeric subunits. Thus, a LT- $\alpha$ 1/ $\beta$ 2 or LT- $\alpha$ 2/ $\beta$ 1 30 molecule may be converted into a CD40 pathway inhibitor.

The receptor binding domain of TNF family ligands is separated from the transmembrane domain by a "stalk" region that has a variable length depending on 35 the family member. The portion of the sequence of the stalk regions for the CD40 ligand is shown in

- 40 -

**Figure 10.** Shorter or longer stalks than those shown in **Figure 10** may also be compatible. Sequence 1 (**Figure 10**) may be constructed in an appropriate vector and the protein co-expressed from that vector along 5 with the wild type soluble LT- $\beta$  protein as described in Example 1. Co-expression can be performed in a suitable system such as the baculovirus/insect cell system described herein, or in another suitable system such as in animal cells or yeasts such as *Pichia* 10 cells.

Chimeric proteins are passed over a CD40 receptor affinity column (made using the same procedure as the LT- $\beta$ -R affinity column in Example 2, except soluble CD40R is coupled to the column), and 15 those proteins possessing a CD40R binding domain are isolated. Complexes possessing a LT- $\beta$  subunit are then purified using an anti-LT- $\beta$  monoclonal affinity column resulting in a preparation of CD40L/LT- $\alpha$  chimera/LT- $\beta$  complexed at a 1:2 and 2:1 ratios. These forms can be 20 separated using another purification step such as ion exchange chromatography. The binding properties of the chimeric complexes to CD40 receptor may then be ascertained using BIACore™ technology as described in Example 5.

25 Improvements in the ability of the CD40/LT- $\alpha$  chimeric subunit to trimerize with LT- $\beta$  subunits can be made at this stage. Using well known mutagenesis procedures, additional amino acids in the CD40 subunit association domain can be changed individually or in 30 groups to increase or decrease further the match between those found in the LT- $\alpha$  subunit association domain. Altered chimeric subunits are then complexed with LT- $\beta$  subunits, and trimers of various compositions separated by affinity chromatography as described 35 above.

- 41 -

Stability of the altered (CD40L/LT- $\alpha$ )<sub>2</sub>/LT- $\beta$ 1 chimeric trimers can be assessed by storing the preparations for extended periods of time and periodically analyzing the samples by ion exchange chromatography to determine the purity of the trimers.

The sequences of the CD40/LT chimeric subunit required to optimize expression, heterotrimer assembly and stability may be determined by making several CD40 forms having increasing lengths of the stalk regions shown in **Figure 10**. If too few of the CD40 stalk sequences are used, proper folding of this region into a  $\beta$ -sandwich structure may be interfered with, thus reducing efficient heterotrimer formation. If these sequences are too long, increased proteolysis could result.

The improved ability of altered chimeric CD40/LT- $\alpha$  subunits to trimerize with LT- $\beta$  subunits may be monitored in parallel with the increase or decrease in CD40 receptor binding activity caused by the length of the CD40 sequences and by additional amino acid alterations.

Similarly, one may also prepare a CD40L/LT- $\beta$  chimeric subunit (**Figure 10; Seq 2**) and co-express it with wild type LT- $\alpha$  subunits. Chimeric (CD40L/LT- $\beta$ )<sub>2</sub>/LT- $\alpha$ 1 heterotrimers that are able to bind to a CD40 Receptor affinity matrix and to an anti-LT- $\alpha$  monoclonal antibody affinity resin can be isolated, characterized and optimized as described above.

Additional mutations may also be incorporated into the LT- $\alpha$  or LT- $\beta$  receptor binding domains of the wild type LT and the chimeric CD40/LT subunits to reduce any residual background binding to TNF-R or LT- $\beta$ -R by (CD40L/LT- $\beta$ )<sub>2</sub>/LT- $\alpha$ 1 or (CD40L/LT- $\alpha$ )<sub>2</sub>/LT- $\beta$ 1 heterotrimers, as described in Example 8.

**35 Fas/LT Chimeric Inhibitors.** A heteromeric FasL/LT- $\alpha$  or FasL/LT- $\beta$  subunit may be made using

- 42 -

sequences shown in **Figure 10** (Seq 3 and Seq 4). Following the same steps outlined above for the CD40/LT chimera, the chimeric heterotrimers having (FasL/LT- $\beta$ )<sub>2</sub>/LT- $\alpha$ 1 and (FasL/LT- $\alpha$ )<sub>2</sub>/LT- $\beta$ 1 stoichiometries may be  
5 constructed, tested and optimized. As described for CD40/LT chimeras, several C-terminal stalk sequences will also be compatible with the expression of a functional molecule. The desired characteristics can be optimized by constructing chimeric FasL/LT subunits  
10 with varying lengths of the C-terminal stalk domains shown in **Figure 10**.

**TNF/LT Chimeric Inhibitors.** A heteromeric TNF/LT- $\alpha$  or TNF/LT- $\beta$  subunit may be constructed using the sequences shown in **Figure 10** (Seq 5 and Seq 6).  
15 Following the same steps outlined above, the chimeric heterotrimers having (TNF/LT- $\beta$ )<sub>2</sub>/LT- $\alpha$ 1 and (TNF/LT- $\alpha$ )<sub>2</sub>/LT- $\beta$ 1 stoichiometries may be constructed, tested and optimized. Also as described above, several C-terminal stalk sequences will also be compatible with  
20 the expression of a functional molecule. The desired characteristics can be optimized by constructing chimeric TNF/LT subunits with varying lengths of the C-terminal stalk domains shown in **Figure 10**.

The stretches of LT- $\alpha$  and LT- $\beta$  sequences that  
25 must be included in a molecule to force the correct heteromeric trimer formation may vary slightly in length for each specific application. By techniques routine to molecular biology, these sequences may be altered by one or two amino acids on each end to form  
30 the optimum structure for a particular chimeric heterotrimer. Likewise, any single amino acid in this scaffold region may not be absolutely crucial to the structure and such minor changes do not constitute unique molecules according to this invention.  
35 Appropriate functional assays for ligand binding

- 43 -

activity will provide the fine tuning enabling one to determine which exact amino acid residues are optimal.

**Altering the Receptor Binding Domains of LT Subunits to Make Chimeric LT Heteromeric Inhibitors**

5       The external amino acid residues that create a receptor binding site have been defined for the binding of LT- $\alpha$  to the p55 TNF-R. Using this structure and the LT- $\alpha$ /LT- $\beta$  heteromer backbone, one can substitute residues from a different ligand (X)

10      predicted to be involved in receptor binding into the equivalent positions of either the LT- $\alpha$  or LT- $\beta$  subunits. One example of a resultant chimeric molecule, LT- $\beta$ /(X) complexed with LT- $\alpha$  in a LT- $\alpha$ 1/ $\beta$ 2 trimer, would now bind receptor X in the LT- $\beta$ (X)/LT-

15       $\beta$ (X) cleft. As only one such cleft exists per molecule, this chimeric trimer inhibits receptor X cross-linking and subsequent signalling.

20      The same manipulations could be carried out on a LT- $\alpha$  scaffold creating LT- $\alpha$ /(X) subunits which, when complexed with LT- $\beta$  in a LT- $\alpha$ 2/ $\beta$ 1 trimer, would also inhibit receptor X signal transduction. Both variations are envisioned in this invention.

25      Receptor binding domains of TNF-like ligands are domains of protein sequence that loop off of the domains which form the TNF-like association sequences. The receptor binding domains comprise specific amino acid sequences that confer the high degree of ligand-receptor binding specificity. Accordingly, exchange of the LT receptor binding loop with one from another

30      ligand of the TNF family would convert the resulting trimeric ligand into one that now binds that different receptor.

35      Once the residues comprising a receptor binding domain of a TNF-like ligand have been identified by analysis of crystal or co-crystal

- 44 -

structures or by structure-function analyses using random and directed mutagenesis, these sequences can be used to replace the corresponding domain of a LT- $\alpha$  or LT- $\beta$  subunit to construct hybrid or chimeric LT- $\alpha/\beta$  trimers with new receptor binding properties. These chimeric LT- $\alpha/\beta$  trimers will function as inhibitors of signalling by the new receptor to which the chimeric trimer binds to.

Homology models of the 3-dimensional structure have been constructed for the human LT- $\beta$  and CD40L molecules. A procedure similar to that described in Greer et al. (Proteins, 7, pp. 317-334 (1990)) was applied making use of the protein modeling tools of the QUANTA (Molecular Simulations, Inc.) program package.

After aligning the sequences of TNF, LT- $\alpha$ , LT- $\beta$  and CD40L, the crystal structure of LT- $\alpha$  was used as a template for the construction of the LT- $\beta$  model. By the same means, the model of the murine CD40L (Peitsch Jongeneel, Int. Immunol., 5, pp. 233- (1993)) was used as a template for the construction of the human CD40L. Loops corresponding to insertions or deletions between the two molecules were selected using fragment search from the Protein Data Bank database. The resulting models were subjected to several cycles of molecular dynamics simulation and energy minimization.

Subsequently, the models were superimposed.

Based on the description of where LT- $\alpha$  and TNF-R contact one another in the receptor-ligand complex (Banner et al., Cell, 73, pp. 431- (1993)), regions of LT- $\beta$  and CD40L chains likely to be involved in specific ligand-receptor interactions have been identified. These regions correspond to loops lying in the cleft formed between monomers. The trimerization interface contains mostly aromatic residues of the large  $\beta$ -sheet and is distinct from the receptor binding domain.

- 45 -

- Based on studies of crystal structures and molecular modeling described above, chimeric LT subunits with heterologous receptor binding loops can be prepared, characterized and optimized. Variations
- 5 to improve upon the receptor "fit" will likely be required and do not represent substantial changes in the scheme claimed herein. These changes can most easily be made in an iterative fashion with currently available technology.
- 10 Using the LT trimeric scaffold, similar loops from other ligands can be readily spliced onto either the LT- $\alpha$  or LT- $\beta$  scaffolds creating --when complexed with the appropriate LT- $\alpha$  or LT- $\beta$  pair --monovalent ligands for these other receptors. For example, to
- 15 make a LT- $\beta$ -CD40L hybrid ligand, the receptor binding loop of the LT- $\beta$  molecule is replaced by the corresponding region from CD40L, which mediates CD40L-CD40 binding. This hybrid subunit would be capable of forming a LT- $\alpha$ 1/ $\beta$ 2 heterotrimer having a single CD40
- 20 binding site in the LT- $\beta$ /LT- $\beta$  cleft. The Fas ligand is closely related to LT- $\alpha$  and LT- $\beta$  and represents an easy extension of the principles outlined above. Similar LT chimeric subunits could potentially be constructed for any TNF-related ligand subunit.
- 25 One can assess the inhibitory potential of these chimeric LT heterotrimers in two ways. First, one can study the strength with which the complexes interact with their cognate receptors. Receptor (i.e. CD40 or Fas) -immunoglobulin chimera could be used for
- 30 conventional binding assays or plasmon resonance detection (Biacore™ technology) as described in Example 5.
- The development and optimization of a receptor binding site could be readily assessed by
- 35 engrafting the receptor binding regions onto LT- $\alpha$ . Since human LT- $\alpha$  can be secreted as a homotrimer, the

- 46 -

chimeric trimer can be expressed and tested directly for binding affinity using Biacore™ technology. Once a good receptor binding site has been created, the chimeric LT- $\alpha$  subunit can be expressed with LT- $\beta$  and 5 the inhibitor heteromeric form isolated. In this way one can optimize the receptor binding site without the need to co-express two genes and perform the purification procedures of distinct heteromeric forms.

Alternatively, inhibitory activity can be 10 measured in a functional assay by measuring the ability of the chimeric inhibitor to block the pathway concerned. For CD40 signalling, one could monitor the ability of the LT/CD40L chimeric construct to induce the death of a cell line containing CD40/TNF chimeric 15 receptors (Hess et al., Eur. J. Immunol., 25, pp. 80-86 (1995)). For Fas signalling, Fas-dependent cell killing assays (Itoh et al., cell, 66, pp. 233-43 (1991)) could be used to measure inhibition of killing with the LT/Fas chimeric construct.

20 These or other binding and inhibitory assays can be used to systematically vary the exact splice points used to form a LT-chimeric subunit. In this way, fine tuning can maximize the effectiveness of LT-chimeric subunits as inhibitors of receptor signalling.

## 25 **Treatments Using LT Heteromeric Inhibitors**

The compositions of this invention will be administered at an effective dose to treat the particular clinical condition addressed. Determination of a preferred pharmaceutical formulation and a 30 therapeutically efficient dose regimen for a given application is well within the skill of the art taking into consideration, for example, the condition and weight of the patient, the extent of desired treatment and the tolerance of the patient for the treatment.

- 47 -

Typically, humans can tolerate up to 100-200 µg/m<sup>2</sup> of TNF before serious toxicity is manifested (Schiller et al., Cancer Res., 51, pp. 1651-58 (1991)). In mice, dosages in the range of 1-5 µg/mouse/day given 5 with 5x10<sup>4</sup> units of recombinant human IFN-γ caused human primary tumor regression (Balkwill et al., CIBA Foundation Symposium (1987); Havell et al., J. Exp. Med., 167, pp. 1067-85 (1988)). Based on the relative effectiveness of TNF and LT-α1/β2 in the HT29 cytolytic 10 assays, approximately 5-25 µg/mouse/day of LT-α1/β2 will provide a therapeutic dose range. Extrapolating to the human, it is expected that LT-α1/β2 and LT-α2/β1 dosages of at least 1 mg/m<sup>2</sup> will be required.

Similar dosages of various altered and 15 chimeric LT heteromeric inhibitors are expected to be suitable starting points for optimizing treatment doses and schedules according to methods known to those of skill in the art.

Administration of the LT-α/β heteromeric 20 inhibitors of this invention, including isolated and purified forms of the complexes, their salts or pharmaceutically acceptable derivatives thereof, may be accomplished using any of the conventionally accepted modes of administration of agents which exhibit anti-tumor activity.

The pharmaceutical compositions used in these therapies may also be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid 30 solutions or suspensions, suppositories, and injectable and infusible solutions. The preferred form depends on the intended mode of administration and therapeutic application. Modes of administration may include oral, parenteral, subcutaneous, intravenous, intralesional or 35 topical administration.

- 48 -

- The LT- $\alpha/\beta$  heteromeric inhibitors may, for example, be placed into sterile, isotonic formulations with or without cofactors which stimulate uptake or stability. The formulation is preferably liquid, or
- 5 5 may be lyophilized powder. For example, the LT heteromeric inhibitors may be diluted with a formulation buffer comprising 5.0 mg/ml citric acid monohydrate, 2.7 mg/ml trisodium citrate, 41 mg/ml mannitol, 1 mg/ml glycine and 1 mg/ml polysorbate 20.
- 10 10 This solution can be lyophilized, stored under refrigeration and reconstituted prior to administration with sterile Water-For-Injection (USP).

The compositions also will preferably include conventional pharmaceutically acceptable carriers well known in the art (see for example Remington's Pharmaceutical Sciences, 16th Edition, 1980, Mac Publishing Company). Such pharmaceutically acceptable carriers may include other medicinal agents, carriers, genetic carriers, adjuvants, excipients, etc., such as

15 20 human serum albumin or plasma preparations. The compositions are preferably in the form of a unit dose and will usually be administered one or more times a day.

The pharmaceutical compositions of this invention may also be administered using microspheres, liposomes, other microparticulate delivery systems or sustained release formulations placed in, near, or otherwise in communication with affected tissues or the bloodstream. Suitable examples of sustained release carriers include semipermeable polymer matrices in the form of shaped articles such as suppositories or microcapsules. Implantable or microcapsular sustained release matrices include polylactides (U.S. Patent No. 3,773,319; EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers, 22, pp. 547-56 (1985)); poly(2-

- 49 -

hydroxyethyl-methacrylate) or ethylene vinyl acetate (Langer et al., J. Biomed. Mater. Res., 15, pp. 167-277 (1981); Langer, Chem. Tech., 12, pp. 98-105 (1982)).

Liposomes containing LT heteromeric

5 inhibitors can be prepared by well-known methods (see, e.g. DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. U.S.A., 82, pp. 3688-92 (1985); Hwang et al., Proc. Natl. Acad. Sci. U.S.A., 77, pp. 4030-34 (1980); U.S. Patent Nos. 4,485,045 and 4,544,545). Ordinarily  
10 the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol.% cholesterol. The proportion of cholesterol is selected to control the optimal rate of LT heteromeric complex release.

15 The LT heteromeric complexes of this invention may also be attached to liposomes which may optionally contain other therapeutic agents directed to the particular treatment being performed. Attachment of LT heteromeric complexes to liposomes may be  
20 accomplished by any known cross-linking agent such as heterobifunctional cross-linking agents that have been widely used to couple toxins or chemotherapeutic agents to antibodies for targeted delivery. Conjugation to liposomes can also be accomplished using the  
25 carbohydrate-directed cross-linking reagent 4-(4-maleimidophenyl) butyric acid hydrazide (MPBH) (Duzgunes et al., J. Cell. Biochem. Abst. Suppl. 16E 77 (1992).

30 **Advantages of therapeutic compositions comprising LT heteromeric inhibitors**

TNF and LT- $\alpha$  are cytokines with an extraordinary range of activities mediated by TNF-R activation. The utility of the LT- $\alpha$ 2/ $\beta$ 1 trimeric molecules stems from their potential to effectively  
35 inhibit TNF-R receptor signalling. The soluble LT-

- 50 -

$\alpha$ 2/ $\beta$ 1 trimers will be useful as therapeutic agents in treating clinical conditions known to be improved by TNF inhibition (Beutler, B., Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine. New York: Raven Press (1994)). Such clinical conditions include septic shock, autoimmune diseases such as diabetes, rheumatoid arthritis, inflammatory bowel disease, and HIV proliferation.

Moreover, the soluble LT- $\alpha$ 2/ $\beta$ 1 inhibitors of this invention comprise human protein sequences, and thus immune recognition by individuals treated with these complexes should be minimal. The complexes of this invention should be amenable to long term therapeutic administration and should be especially suitable for treating chronic autoimmune conditions.

The utility of the LT- $\alpha$ 1/ $\beta$ 2 inhibitors stems from their potential to effectively inhibit LT- $\beta$ -R receptor signalling. In mice, disruption of the LT- $\alpha$  gene leads to aberrant peripheral lymphoid organ development (De Togni et al., *Science*, 264, pp. 703-7 (1994)). We believe that this phenotype is associated with loss of LT- $\beta$ -R signalling because no similar phenotypes have been observed by modulating TNF-R activity.

Substitution of the binding cleft residues to create chimeric trimers which bind to receptors other than p55 TNF-R, p75 TNF-R or LT- $\beta$ -R will also have clinical relevance in treating diseases or conditions by inhibiting signalling pathways which utilize those other specific receptors.

For example, in one embodiment of this invention, signalling through the CD40 pathway is inhibited using chimeric LT heterotrimers that have been converted into monovalent CD40R-specific ligands. Signalling through the CD40 pathway induces B cells, upon stimulation by helper T cells, to undergo

- 51 -

immunoglobulin (Ig) maturation and secretion. Immature B cells begin by making only surface IgM. Upon signals from an activated helper T cell, B cells proliferate and mature into memory and antibody-secreting cells.

- 5 The signal responsible for this maturation process is initiated by CD40R signalling. Thus the LT/CD40 chimeric ligands of the present invention could be useful both for modulating certain Ig maturation steps during B cell development, and for inhibiting the onset  
10 or controlling the severity of certain autoimmune diseases in humans.

One preferred embodiment of this invention is directed to chimeric CD40/LT subunits which comprise LT subunit association sequences inserted or engineered  
15 into CD40 ligand polypeptides which have CD40 receptor binding domains. The LT subunit association sequences can drive complex formation between LT and CD40/LT chimeric subunits. The sequence conservation within  
20 TNF homology regions of the TNF ligand family, and the apparent conservation of the  $\beta$ -sandwich structures which can form from the TNF homology domains, permits the design of CD40, Fas, TNF, and other such chimeric LT ligands. It is thus envisioned that similar  
25 chimeric LT complexes may be made by applying the techniques described herein to other ligand polypeptides belonging to the TNF family. Coupled with the powerful immunological techniques which are currently available, such as those described herein,  
functional chimeric molecules can be selected from a  
30 mixture of various chimeric heteromeric complexes and can be identified and purified in a straight-forward manner.

Another preferred embodiment of this invention is directed to the use of CD40L sequences to  
35 alter receptor binding properties of LT- $\alpha/\beta$  trimers to inhibit the CD40 pathway. Although this embodiment, as

- 52 -

well as the one described above, is directed to making CD40/LT chimeric ligands, the invention as envisioned is in no way limited to this one example. Any region of a ligand which is capable of conferring receptor binding affinity and which can be inserted onto the LT- $\alpha/\beta$  trimers scaffold in such a way that it alters or converts receptor binding characteristics of a resulting heteromeric ligand is anticipated to fall within the scope of this invention.

Another embodiment of this invention involves inhibiting signalling through the Fas pathway using chimeric Fas/LT heterotrimers that have been converted into monovalent Fas-specific ligands. As discussed above, signalling through the Fas pathway has pleiotropic effects. Activated Fas can kill cells by apoptosis. Apoptosis is responsible for negative thymocyte selection during normal T cell maturation, as well as for mature T cell-mediated cytotoxicity by killer T cells. Cell killing mechanism is a major component of graft rejection and therefore inhibitors of the Fas pathway may be useful in reducing tissue rejection following organ transplantation. Fas signalling is also involved in the T cell apoptosis observed during HIV disease (Katsikis et al., J. Exp. Med., 181, pp. 2029-36 (1995)). Inhibitors of the Fas pathway may also be useful in blocking HIV-induced lymphocyte depletion.

In mice, naturally-occurring recessive mutations in both the Fas and FasL genes have been identified. Both cause lymphoproliferation diseases, including lymphadenopathy, and an autoimmune disease similar to systemic lupus erythematosus in humans. Recent studies in these mice suggest that Fas signalling is involved in apoptosis of chronically-activated mature T cells, and suggest a role for Fas

- 53 -

signalling in peripheral tolerance (Russel and Wang, Eur. J. Immunol., 23, pp. 2379-82 (1993)).

Fresh primary B thymocytes (PBT) cells and some B cell tumors respond to Fas activation by 5 proliferating rather than by apoptotic death (Mapara et al., Eur. J. Immunol., 23, pp. 702-8 (1993); Alderson et al., J. Exp. Med., 178, pp. 2231-35 (1993)). In these instances, inhibition of Fas signalling by monovalent Fas/LT chimeric ligands could be useful in 10 inhibiting deleterious B cell proliferation.

In addition, signalling through the Fas receptor is thought to be involved in liver necrosis during fulminant hepatitis (Ogasawara et al., Nature, 364, pp. 806-809 (1993)). Blockage of this signalling 15 pathway may be beneficial in reversing or slowing down the destruction of liver tissue in such instances.

The pleiotropic nature of Fas signalling is a feature of several of the other TNF family members, including CD30 and both TNF receptors. In each case, 20 the specific effect of ligand-induced receptor signalling, including cell death by apoptosis or necrosis, or cell proliferation, is dependent on the cell type, its stage of differentiation, its transformation status and on other environmental factors (Smith et al., Cell, 76, pp. 959-62 (1994)). It is thus expected that new effects of signalling 25 through various TNF and TNF-like receptors will continue to be found.

As known receptors become linked to various 30 medical disorders, and as new signalling pathways and new receptors are discovered, the chimeric LT heterotrimeric complexes of the present invention will be useful as inhibitors of these signalling pathways by applying the methods taught herein.

35 The following are examples which illustrate the LT heteromeric inhibitors of this invention and the

- 54 -

methods used to characterize them. These examples should not be construed as limiting: the examples are included for purposes of illustration and the present invention is limited only by the claims.

5

**Example 1**  
**Generation of Baculovirus-infected Insect Cell Supernatants Containing LT- $\alpha$ / $\beta$  Forms**

Recombinant baculovirus was prepared encoding either full length LT- $\alpha$  or a secreted form of LT- $\beta$  as described (Crowe et al., J. Immunol. Methods, 168, pp. 79-89 (1994)). High five™ insect cells were inoculated at a density of  $2 \times 10^5$  cells/ml into 7.2 liters of SF 900-II (Gibco) media without serum. The culture reached  $1.8 \times 10^6$  cells/ml 48 hours later and was infected with 150 ml ( $3 \times 10^8$  PFU/ml) LT- $\beta$  and 300 ml of LT- $\alpha$  baculovirus stocks. Two days later, the culture was harvested and the cell debris was removed by centrifugation. After addition of 1 mM EDTA and 150 uM PMSF (final concentration), the clarified supernatant was concentrated 10 fold by ultrafiltration using a S1YM10 (Amicon) spiral cartridge. The concentrate was divided into six 120 ml portions and aliquots were stored at -70°C prior to purification.

25

**Example 2**  
**Purification of LT- $\alpha$ 2/ $\beta$ 1 using p55 TNF-R and LT- $\beta$ -R**

Receptor affinity reagents can be prepared as either a soluble extracellular domain (see for example Loetscher et al., J. Biol. Chem., 266, pp. 18324-29 (1991)), or as chimeric proteins with the extracellular ligand binding domain coupled to an immunoglobulin Fc domain (Loetscher et al., J. Biol. Chem., 266, pp. 18324-29 (1991); Crowe et al., Science, 264, pp. 707-710 (1994)). Receptors are coupled to affinity matrices by chemical cross-linking using routine procedures.

- 55 -

To prepare soluble LT- $\beta$ -R/Immunoglobulin Fc chimera, the extracellular domain of LT- $\beta$ -R up to the transmembrane region was amplified by PCR from a cDNA clone using primers that incorporated NotI and SalI  
5 restriction enzyme sites on the 5' and 3' ends, respectively (Browning et al., J. Immunol., 154, pp. 33-46 (1995)). The amplified product was cut with NotI and SalI, purified and ligated into NotI-linearized vector pMDR901 along with a SalI-NotI fragment encoding  
10 the Fc region of human IgG1. The resultant vector contained the dihydrofolate reductase gene and the LT- $\beta$ -R-Fc chimera driven by separate promoters.

The vector was electroporated into CHO dhfr<sup>-</sup> cells and methotrexate-resistant clones were isolated  
15 as per standard procedures. The LT- $\beta$ -R-Fc is secreted into the medium and an ELISA assay was used to select for cell lines producing the highest level of the chimeric protein. A high-producing cell line was grown to large numbers and conditioned medium collected. The  
20 pure protein was isolated by Protein A sepharose fast flow affinity chromatography.

To prepare resins for the receptor affinity purification of LT forms, purified preparations of LT- $\beta$ -R-Fc and p55-Fc (Crowe et al., Science, 264, pp. 25 707-710 (1994))) were immobilized on CNBr-sepharose (Pharmacia) at 5 mg/ml resin following the manufacturer's specifications. The resins were put through one elution cycle prior to use. A portion (120 ml) of the S1Y10 concentrate was passed over a p55 TNF-R-Fc column and the flow through was saved for the purification of LT- $\alpha$ 1/ $\beta$ 2 (see below). The column was washed sequentially with 5 column volumes of phosphate buffered saline (PBS), PBS with 0.5 M NaCl and PBS and then eluted with 25 mM sodium phosphate, 100 mM NaCl,  
30 pH 2.8. Elution fractions were immediately neutralized with 1/20 volume of 0.5 M sodium phosphate, pH 8.6 and  
35

- 56 -

stored on ice. Fractions containing protein were identified by absorbance at 280 nm, pooled and the elution pools from the six columns were analyzed by SDS-PAGE stained with coomassie brilliant blue. The 5 p55 TNF-R-FC column eluate contained purified LT- $\alpha$ 2/ $\beta$ 1 and LT- $\alpha$  and the mix was further purified by affinity purification over a LT- $\beta$ -R-Fc column. The LT- $\alpha$  flows through this column and the LT- $\alpha$ 2/ $\beta$ 1 is retained. Elution as described above yielded pure LT- $\alpha$ 2/ $\beta$ 1.

10

**Example 3**

**Purification of LT- $\alpha$ 1/ $\beta$ 2 using p55 TNF-R and LT- $\beta$ -R**

A portion (120 ml) of the S1Y10 concentrate was passed over two sequential p55 TNF-R-Fc column as described above and the flow through which was now 15 depleted of LT- $\alpha$  and LT- $\alpha$ 2/ $\beta$ 1 was further passed over a LT- $\beta$ -R-Fc column. The column was washed with 5 volumes each of PBS, PBS with 0.5 M NaCl and PBS and eluted with 25 mM sodium phosphate, 100 mM NaCl, pH 3.5. Elution fractions were immediately neutralized with 20 1/20 volume of 0.5 M sodium phosphate, pH 8.6 and stored on ice. Fractions containing protein were identified by absorbance at 280 nm, pooled and the elution pools from the columns were analyzed by SDS-PAGE stained with coomassie brilliant blue. 25 Elution as described above yielded pure LT- $\alpha$ 1/ $\beta$ 2.

**Example 4**

**Characterization of the Purified LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1 Ligands**

The stoichiometry of LT- $\alpha$  to LT- $\beta$  contained 30 in the purified LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1 fractions was evaluated either by densitometry tracing of the coomassie stained gels or by peak height analysis of the two peaks obtained following resolution on C4 reverse phase HPLC. The purified fractions were sized

- 57 -

by gel exclusion chromatography to assess whether trimers were formed and whether aggregates were present. A TSK G2000 sw xl column was used at a flow rate of 0.5 ml/min to size separate a BioRad gel 5 filtration protein standard, and the three different LT trimers: LT- $\alpha$ 3, LT- $\alpha$ 2/ $\beta$ 1 and LT $\alpha$ 1/ $\beta$ 2. Very little, if any, of the three trimers behave as high molecular weight aggregates (**Figure 4a**). Comparison to size standards shows that the three forms are all trimeric, 10 i.e., about 50-60 kDa. Given the trimeric size and the C4 reverse phase HPLC data (**Figure 3**), the only possible composition for these preparations can be LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1.

The purity of the preparations was assessed 15 further by ion exchange chromatography. BioCAD™ instrumentation was utilized to run pH maps of LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1 on the weak cation exchanging resin under several different buffer systems. The method that exhibited the greatest ability to cleanly retain and 20 separate the three trimers incorporated a POROS CM (carboxymethyl) column run at 5 ml/min in a 16.66 mM MES, 16.66 mM HEPES, 16.66 mM Sodium acetate buffer (pH 6.5) and eluting with a 1 M NaCl gradient over 20 column volumes. The BioCAD™ chromatograms of LT- $\alpha$ 1/ $\beta$ 2 25 and LT- $\alpha$ 2/ $\beta$ 1 forms is shown in **Figure 4b**. Each trimer (LT- $\alpha$ 3, LT- $\alpha$ 2/ $\beta$ 1 and LT- $\alpha$ 1/ $\beta$ 2) eluted at a different salt concentration and there was no evidence for cross contamination of more than 1-2% in the various preparations. These biochemical analyses confirm that 30 the purification schemes outlined in Examples 2 and 3 yield the expected purified LT heterotrimers.

- 58 -

**Example 5**  
**Assessment of the Binding Interaction Between LT Heterotrimeric Ligands and LT- $\beta$  and TNF Receptors**

The plasmon resonance detection method as  
5 embodied by the BIACore™ instrument (BioSensor) provides a powerful method for the determination of the strength of binding interactions between two proteins (Zhou et al., *Biochemistry*, 32, pp. 8193-98 (1993); Faegerstram and O'Shannessy, "Surface plasmon resonance  
10 detection in affinity technologies". In Handbook of Affinity Chromatography, pp. 229-52, Marcel Dekker, Inc., New York (1993)). Typically, a soluble receptor, e.g. p55TNF-R-immunoglobulin Fc fusion protein, is bound to the "chip" supplied by the manufacturer using  
15 various known chemistries. In this case, the N-hydroxy-succinimide based linkage system was employed according to the specifications of the manufacturer. The coupled chip was washed in the instrument, and soluble ligand at various concentrations between 0.1  
20 and 100 µg/ml was flowed over the chip. Bound protein is directly indicated by the instrument. **Figure 5a** shows an example of the sensorgrams depicting the binding of LT- $\alpha$ 1/ $\beta$ 2 and LT- $\alpha$ 2/ $\beta$ 1 to the LT- $\beta$  receptor.

BIACore™ technology allows one to bind  
25 receptor to a gold surface and ligand is flowed over it. Plasmon resonance detection gives direct quantitation of the amount of mass bound to the surface in real time. This technique yields both on and off rate constants and therefore the Kd (dissociation  
30 constant) can be directly determined, and affinity constants for each interaction can be assessed. **Figure 5b** shows an example of the sensorgrams depicting the binding of LT- $\alpha$  and LT- $\alpha$ 2/ $\beta$ 1 to the p55 TNF-R.

From the supplied software, one obtains on  
35 and off rates for each tested interaction. The equilibrium binding constant is the ratio of the on to

- 59 -

off binding rates. Thus if one wanted to create a LT heteromeric ligand having a tighter receptor binding site, one would make alterations in one or more amino acid residues in the receptor binding domain of the  
5 ligand subunits that resulted in either an increased on rate or a decreased off rate.

Likewise, to make a weaker receptor binding site, one would make alterations that decreased the on rate or increased the off rate of the LT heteromeric  
10 ligand to the receptor. A number of conventional methods to assess ligand-receptor binding, such as ELISA based assays could also be used to obtain such information.

**Example 6**

**Inhibition of TNF activity by Purified LT- $\alpha$ 2/ $\beta$ 1**

Mouse L929 cells can be killed by TNF or LT- $\alpha$ 3 in either short term cytolytic assays or in longer term growth inhibition assays. These assays have been described (Browning et al., J. Immunol., 143, 20 pp. 1859-67 (1989)). Purified LT- $\alpha$ 2/ $\beta$ 1 can inhibit the cytolytic activity of TNF or LT- $\alpha$ 3 in a number of culture systems. **Figure 6a** shows a typical competition assay showing that LT- $\alpha$ 2/ $\beta$ 1 can effectively compete with TNF in the mouse L929 cytolytic assay.

The HT29 human adenocarcinoma cytolytic assay has been previously described (Browning and Ribolini, J. Immunol., 143, pp. 1859-67 (1989)). This protocol can also be used to measure growth inhibition of other tumor lines, such as the Me 180 cells used here.  
30 Interferon- $\alpha$  was included in tumor cell assays since it acts synergistically with TNF to inhibit tumor cell growth.

In a typical assay, serial dilutions of LT- $\alpha$ 2/ $\beta$ 1 were prepared in 0.05 ml in 96 well plates and  
35 5000 trypsinized HT29 cells added in 0.05 ml of media

- 60 -

containing 80 U/ml (anti-viral units) of human IFN- $\gamma$ .  
(HT29-14 cells, which are from a subclone of the  
original ATCC-derived HT29 line that is more  
homogeneous give the same results as the original ATCC-  
derived HT29 line).

After 3-4 days, mitochondrial reduction of  
the dye MTT was measured as follows: 10  $\mu$ l of MTT was  
added and after 3 hours, the reduced dye dissolved with  
0.09 ml of isopropanol with 10mM HCl, and the O.D.  
measured at 550 nm. The number of viable cells is  
proportional to the absorbance.

The ability of LT- $\alpha$ 2/ $\beta$ 1 to inhibit the  
cytotoxic effects of TNF in a typical growth inhibition  
assay using the human tumor lines Me180 and HT29 are  
shown in **Figures 6b and 6c**, respectively, and are  
performed using essentially the same assays.

**Example 7**  
**Inhibition of the Binding of LT- $\beta$ -R to Surface LT- $\alpha$ / $\beta$   
by Purified LT- $\alpha$ 1/ $\beta$ 2 or LT- $\alpha$ 2/ $\beta$ 1**

The ability of a soluble LT heteromeric  
ligand, and of altered or chimeric forms thereof, to  
block signalling through the LT- $\beta$  receptor can be  
tested in cell surface binding competition assays.  
Human activated T cell hybridoma II-23 cells express  
surface LT- $\alpha$ 1/ $\beta$ 2 upon PMA activation (Browning et al.,  
*Cell*, 72, pp. 847-56 (1993)). By mixing these cells  
with varying concentrations of the soluble ligand and  
then adding soluble receptor (LT- $\beta$ -R-Fc), one can  
measure how well the soluble ligand can compete with  
the cell surface ligand for binding to the soluble  
receptor. Using an anti-human IgG antibody coupled to  
a fluorophore (phycoerythrin was used here), one can  
quantitate bound receptor using FACS analysis (Crowe et  
al., *Science*, 264, pp. 707-710 (1994)) following the  
binding of soluble LT- $\beta$ -R-Fc chimera to LT- $\alpha$ 1/ $\beta$ 2 on the

- 61 -

surface of PMA-activated II-23 cells. This assay is a useful tool in the design of altered LT- $\alpha$ 1/ $\beta$ 2 forms that retain LT- $\beta$  receptor binding activity, yet lack the ability to induce receptor signalling.

- 5       **Figure 7** shows the ability of various amounts of soluble ligand to block receptor binding using the assay described above. A reduction in the mean fluorescence value was used to calculate percent inhibition. Molecular weights of 90 kDa for the  
10 dimeric receptor-Fc construct and 65kDa for the trimeric ligand were employed to calculate molarities. Binding competition was carried out for 30 minutes on ice followed by washing of the cells and probing of bound human Fc with a secondary antibody.

15       **Example 8**  
**Mutagenesis of Ligand Subunits to Eliminate Background Receptor Signalling by LT Heteromeric Inhibitors**

- To eliminate the trace cytolytic activity caused by TNF-R signalling, an Asp50 to Asn50 mutation  
20 was introduced into the LT- $\alpha$  construct using "U.S.E." mutagenesis (Pharmacia). A recombinant baculovirus capable of expressing the "LT- $\alpha$ (D50N)" mutant was prepared and insect cells were co-infected with the LT- $\alpha$ (D50N) and wild-type LT- $\beta$  viruses. The insect cell  
25 supernatant was passed over a LT- $\beta$ -R affinity column, and the bound fraction eluted as described in Example 3. This fraction contained both LT- $\alpha$ (D50N)1/ $\beta$ 2 and LT- $\alpha$ (D50N)2/ $\beta$ 1 forms. These forms were separated from each other by ion exchange chromatography as described  
30 in Examples 2 and 3, yielding purified LT- $\alpha$ (D50N)1/ $\beta$ 2 and LT- $\alpha$ (D50N)2/ $\beta$ 1 modified heterotrimers.

**Figure 8a** shows the growth inhibition activity of wild-type LT- $\alpha$ 1/ $\beta$ 2 and mutant LT- $\alpha$ (D50N)1/ $\beta$ 2 trimers on WEHI 164 cells. Normal  
35 preparations of LT- $\alpha$ 3 trimers show 50% growth

- 62 -

inhibition at 1-10 pg/ml, and wild-type LT- $\alpha$ 1/ $\beta$ 2 preparations typically retain 0.1-1% of an LT- $\alpha$ -like activity. The D50N mutation, which abolishes activity of normal LT- $\alpha$ 3 on these cells, also eliminated the 5 residual LT- $\alpha$ -like activity in the altered LT- $\alpha$ 1/ $\beta$ 2 preparation.

**Figure 8b** shows that the LT- $\alpha$ (D50N)1/ $\beta$ 2 altered heteromeric complex retains full specific activity in a WiDr cytotoxicity assay (performed as per 10 the HT29 assay in Browning and Ribolini, J. Immunol., 143, pp. 1859-67 (1989)), which measures signalling through the LT- $\beta$ -R. A similar WiDr assay is described in applicants' co-pending United States application Serial no. 08/378,968. A series of tissue culture 15 wells containing tumor cells such as WiDr cells are cultured for three to four days in media containing IFN- $\gamma$  and either wild type or the mutant LT- $\alpha$ (D50N)1/ $\beta$ 2 heteromeric complex to be tested. A vital dye stain which measures mitochondrial function (such as MTT) is 20 added to the cell mixture and reacted for several hours, and then the optical density of the mixture in each well is quantitated at 550 nm wavelength light (OD 550). The OD 550 is proportional to the growth of tumor cells in the presence of the LT- $\alpha$ / $\beta$  heteromeric 25 complex.

- 63 -

Claims

We claim:

1. A LT- $\alpha/\beta$  heteromeric complex comprising at least one LT- $\alpha$  subunit in association with at least one LT- $\beta$  subunit, wherein at least two LT subunits comprise a receptor binding domain, and wherein each LT subunit comprises a subunit association domain capable of associating with at least one other subunit association domain to form the complex.
2. The LT- $\alpha/\beta$  heteromeric complex according to claim 1, wherein the complex is soluble.
3. The LT- $\alpha/\beta$  heteromeric complex according to claim 2, wherein each LT- $\beta$  subunit has an N-terminus at any amino acid residue between about 44 and 88, thereby increasing the solubility of the complex.
4. The LT- $\alpha/\beta$  heteromeric complex according to claim 1, having a subunit stoichiometry of LT- $\alpha_2/\beta_1$ .
5. The LT- $\alpha/\beta$  heteromeric complex according to claim 1, having a subunit stoichiometry of LT- $\alpha_1/\beta_2$ .
6. The LT- $\alpha/\beta$  heteromeric complex according to claim 1, wherein at least one LT- $\alpha$  receptor binding domain has been mutated at one or more amino acid residues to alter the binding affinity of the complex for TNF-R without substantially disrupting association between the LT subunits of the complex.
7. The LT- $\alpha/\beta$  heteromeric complex according to claim 6, wherein at least one LT- $\alpha$  receptor binding domain has been mutated at amino acid D50 to reduce LT- $\alpha$  binding to TNF-R.

- 64 -

8. The LT- $\alpha/\beta$  heteromeric complex according to claim 1, wherein at least one LT- $\beta$  receptor binding domain has been mutated at one or more amino acid residues to alter the binding affinity of the complex for LT- $\beta$ -R without substantially disrupting association between the LT subunits of the complex.

9. The LT- $\alpha/\beta$  heteromeric complex according to claim 4, wherein at least one amino acid residue in the LT- $\alpha$  subunit receptor binding domain has been exchanged with at least one different amino acid residue of a receptor binding domain from a TNF-related ligand other than the LT- $\alpha$  subunit to alter the receptor binding characteristics of the complex without substantially disrupting association between the LT subunits of the complex.

10. The LT- $\alpha/\beta$  heteromeric complex according to claim 9, wherein the TNF-related ligand is selected from the group consisting of CD40L, FasL and TNF.

11. The LT- $\alpha/\beta$  heteromeric complex according to claim 5, wherein at least one amino acid residue in the LT- $\beta$  subunit receptor binding domain has been exchanged with at least one different amino acid residue of a receptor binding domain from a TNF-related ligand other than the LT- $\beta$  subunit to alter the receptor binding characteristics of the complex without substantially disrupting association between the LT subunits of the complex.

12. The LT- $\alpha/\beta$  heteromeric complex according to claim 11, wherein the TNF-related ligand is selected from the group consisting of CD40L, FasL and TNF.

- 65 -

13. A LT heterotrimeric complex comprising one LT subunit in association with two chimeric ligand subunits, wherein at least the two chimeric subunits comprise a receptor binding domain, and wherein each subunit comprises a subunit association domain capable of associating with at least one other subunit association domain to form the complex.

14. The LT heterotrimeric complex according to claim 13, wherein the complex is soluble.

15. The LT heterotrimeric complex according to claim 13, wherein the LT subunit is an LT- $\alpha$  subunit selected from the group consisting of lymphotoxin- $\alpha$ , native human or animal lymphotoxin- $\alpha$ , recombinant lymphotoxin- $\alpha$ , soluble lymphotoxin- $\alpha$ , secreted lymphotoxin- $\alpha$ , lymphotoxin- $\alpha$  muteins, or lymphotoxin- $\alpha$ -active fragments of any of the above.

16. The LT heterotrimeric complex according to claim 15, wherein the LT- $\alpha$  subunit comprises a receptor binding domain.

17. The LT heterotrimeric complex according to claim 16, wherein the LT- $\alpha$  receptor binding domain has been mutated at one or more amino acid residues to alter the binding affinity of the complex for TNF-R without substantially disrupting association between the subunits of the complex.

18. The LT heterotrimeric complex according to claim 13, wherein the LT subunit is an LT- $\beta$  subunit selected from the group consisting of lymphotoxin- $\beta$ , native human or animal lymphotoxin- $\beta$ , recombinant lymphotoxin- $\beta$ , soluble lymphotoxin- $\beta$ , secreted

- 66 -

lymphotoxin- $\beta$ , lymphotoxin- $\beta$  muteins, or lymphotoxin- $\beta$ -active fragments of any of the above.

19. The LT heterotrimeric complex according to claim 18, wherein the LT- $\beta$  subunit comprises a receptor binding domain.

20. The LT heterotrimeric complex according to claim 19, wherein the LT- $\beta$  receptor binding domain has been mutated at one or more amino acid residues to alter the binding affinity of the complex for LT- $\beta$ -R without substantially disrupting association between the subunits of the complex.

21. The LT heterotrimeric complex according to claim 13, wherein each chimeric subunit comprises a receptor binding domain which can bind to a TNF-related receptor.

22. The LT heterotrimeric complex according to claim 21, wherein the TNF-related receptor is selected from the group consisting of CD40-R, Fas and TNF-R.

23. A pharmaceutical composition comprising a therapeutically effective amount of a LT- $\alpha/\beta$  heteromeric complex according to any one of claims 1-12, and a pharmaceutically acceptable carrier.

24. A pharmaceutical composition comprising a therapeutically effective amount of a LT heterotrimeric complex according to any one of claims 13-22, and a pharmaceutically acceptable carrier.

25. A method for preventing or reducing the severity of an autoimmune disease comprising the step

- 67 -

of administering a therapeutically effective amount of a pharmaceutical composition according to claim 23.

26. A method for preventing or reducing the severity of an autoimmune disease comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 24.

27. A method for preventing or reducing the severity of an immune response to a tissue graft comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 23.

28. A method for preventing or reducing the severity of an immune response to a tissue graft comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 24.

29. A method for treating a clinical condition associated with TNF-R signalling comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 23, wherein the LT- $\alpha/\beta$  heteromeric complex is capable of inhibiting TNF-R signalling.

30. A method for treating a clinical condition associated with TNF-R signalling comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 24, wherein the LT heterotrimeric complex is capable of inhibiting TNF-R signalling.

31. A method for treating a clinical condition associated with LT- $\beta$ -R signalling comprising

- 68 -

the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 23, wherein the LT- $\alpha/\beta$  heteromeric complex is capable of inhibiting LT- $\beta$ -R signalling.

32. A method for treating a clinical condition associated with LT- $\beta$ -R signalling comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 24, wherein the LT heterotrimeric complex is capable of inhibiting LT- $\beta$ -R signalling.

33. A method for treating a clinical condition associated with TNF-related receptor signalling comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 23, wherein the LT- $\alpha/\beta$  heteromeric complex is capable of inhibiting the TNF-related receptor signalling.

34. The method according to claim 33, wherein the TNF-related receptor is selected from the group consisting of CD40-R, Fas and TNF-R.

35. A method for treating a clinical condition associated with TNF-related receptor signalling comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 24, wherein the LT heterotrimeric complex is capable of inhibiting signalling by the TNF-related receptor.

36. The method according to claim 35, wherein the TNF-related receptor is selected from the group consisting of CD40-R, Fas and TNF-R.

- 69 -

37. A method for inhibiting signalling by a TNF-related receptor comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 23, wherein the LT- $\alpha/\beta$  heteromeric complex is capable of inhibiting the TNF-related receptor signalling.

38. The method according to claim 37, wherein the TNF-related receptor is selected from the group consisting of TNF-R, LT- $\beta$ -R, CD40-R and Fas.

39. A method for inhibiting signalling by a TNF-related receptor comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to claim 24, wherein the LT heterotrimeric complex is capable of inhibiting signalling by the TNF-related receptor.

40. The method according to claim 39, wherein the TNF-related receptor is selected from the group consisting of TNF-R, LT- $\beta$ -R, CD40-R and Fas.

1/12

hTNF	76	RTPSDKP--VAHVVANPQAEQGQ--LQWLNR
hLT- $\alpha$	56	AHSTLKP--AAHLIGDPSK--ONSLLWRAA
hLT- $\beta$	82	DLSPGLP--AAHLIGAPLK-GOG-LGWETT
hFasL	139	BKKELRK--VAHLIGKSNSRSMP-LEWEDE
hCD40L	114	QKGDQNQPQIAAHVISEASSKTTSVLQWAEK

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hTNF	RANALLAN-GVELRD--NQ-LVVPSEGGLYIY-SQVLFKGQQCP-----STHV-LLHTHTISRIAVSYOTKVN--LLSAIKSPCQRET--PEGAEAK
hLT- $\alpha$	TDR AFLQD-GFSL SLS--NNS-LLVPTSGIYFVYNSQVYFSGKAY-SPKAT-S SPL-YLAHEVQLFSQYPFHVP--LLSOKM---VY---PGLQE--
hLT- $\beta$	KEQAF LT-SGTQ FSDAEG--LALPQDG LYIYTCLVGYRGRAPPGGDPQGRS VTLRSLYRAGGA-YGP GTPELLEG AETVTPVLD PARR QGYG-
hFasL	YGIVL LS--GVKYKK-GG--LVINETG LYFVY-SKVYFRGQSCNMNOPLSHKVYMRN---SK---Y PQ---DLVMMEGKMM---SY---CTTGQ-
hCD40L	-GYYTMSNNLWVVTL-ENGKQ-LTVKRQGLYYIYA-QVT F-----CSNREASSQAPFIASLCLXSPGRFE-R-I--LLRAAN---THSSAKPC--GQ-

hTNF	P-WYEPIYLGGVFOLEKGDRILSAEINRPDYLDFAESGQVY--FGII AL	233	(PENNICA ET AL, 1984)
hLT- $\alpha$	P-WLHSMYHGA AFQLTQGDQLSTHTD GIPHI VLSP ST-V E--FGAF AL	205	(GRAY ET AL, 1984)
hLT- $\beta$	PLWYTSVGF GGLVQLRRGERVYVNISHPD MVDFARGK-TF--FGAV MG	244	(BROWNING ET AL, 1993)
hFasL	-MWARSSYI LGAVFNLTSADHLYVNVS ELSI VNFE SSQ-TF--FG LYKL	281	(TAKAHASHI ET AL, 1994)
hCD40L	---QS IHLGGV FELQPGASV FVN VTDP SQV--SHGTG-FTSF GLLKL	261	(HOLLENBAUGH ET AL, 1992)

NUMBERING GIVEN IN INDICATED REFERENCE.

FIG. 1

2/12

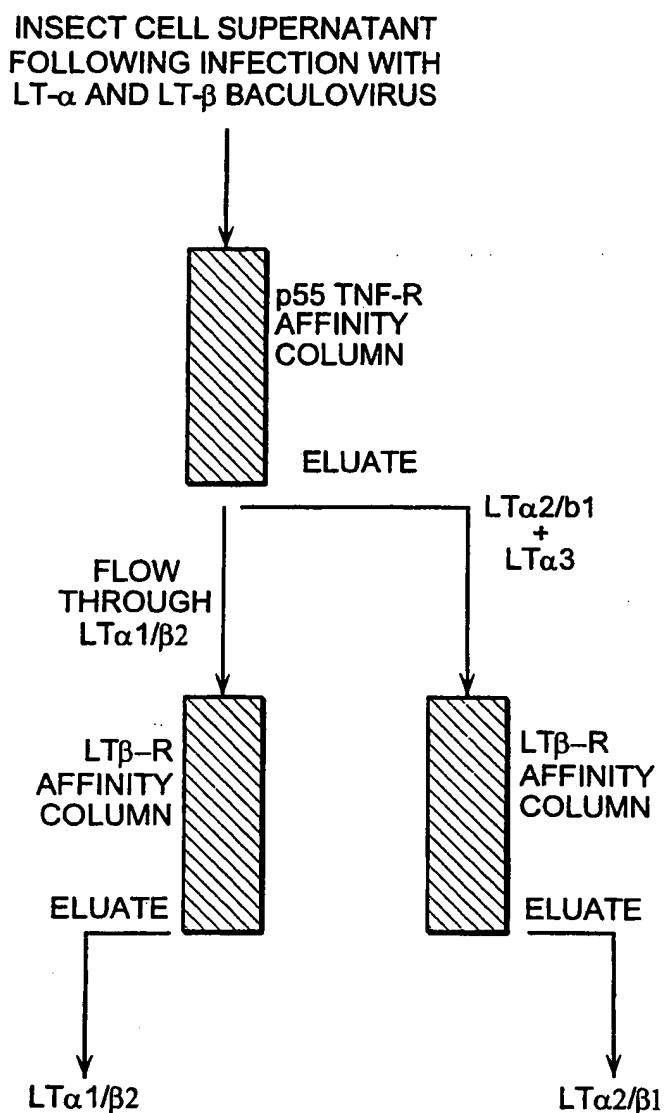


FIG. 2

3/12

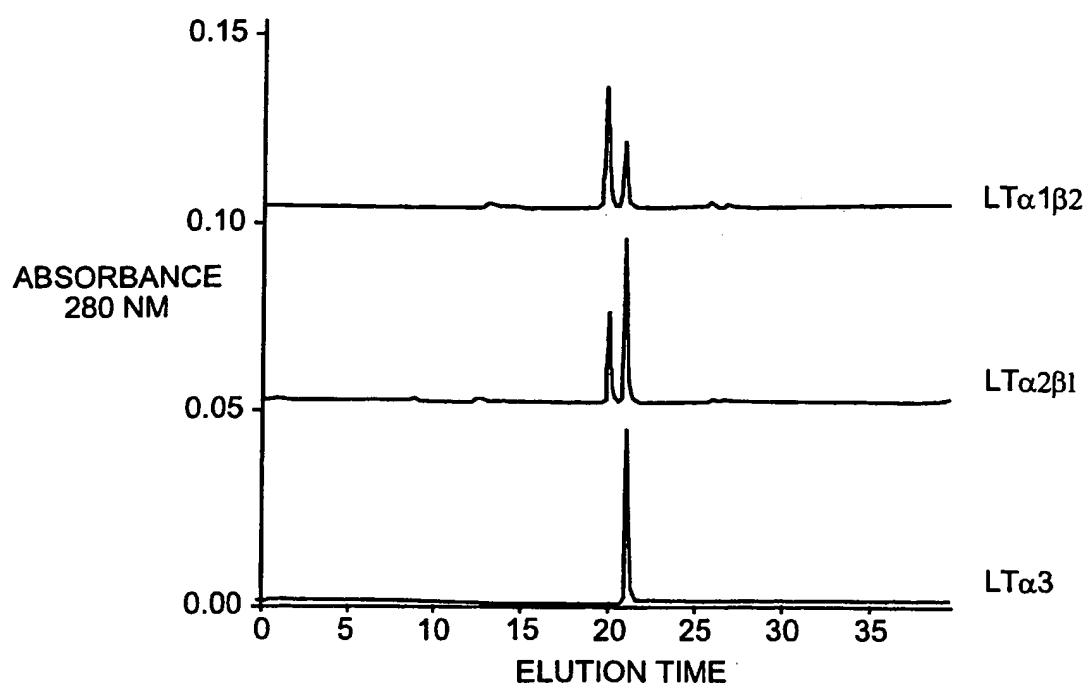


FIG. 3

4/12

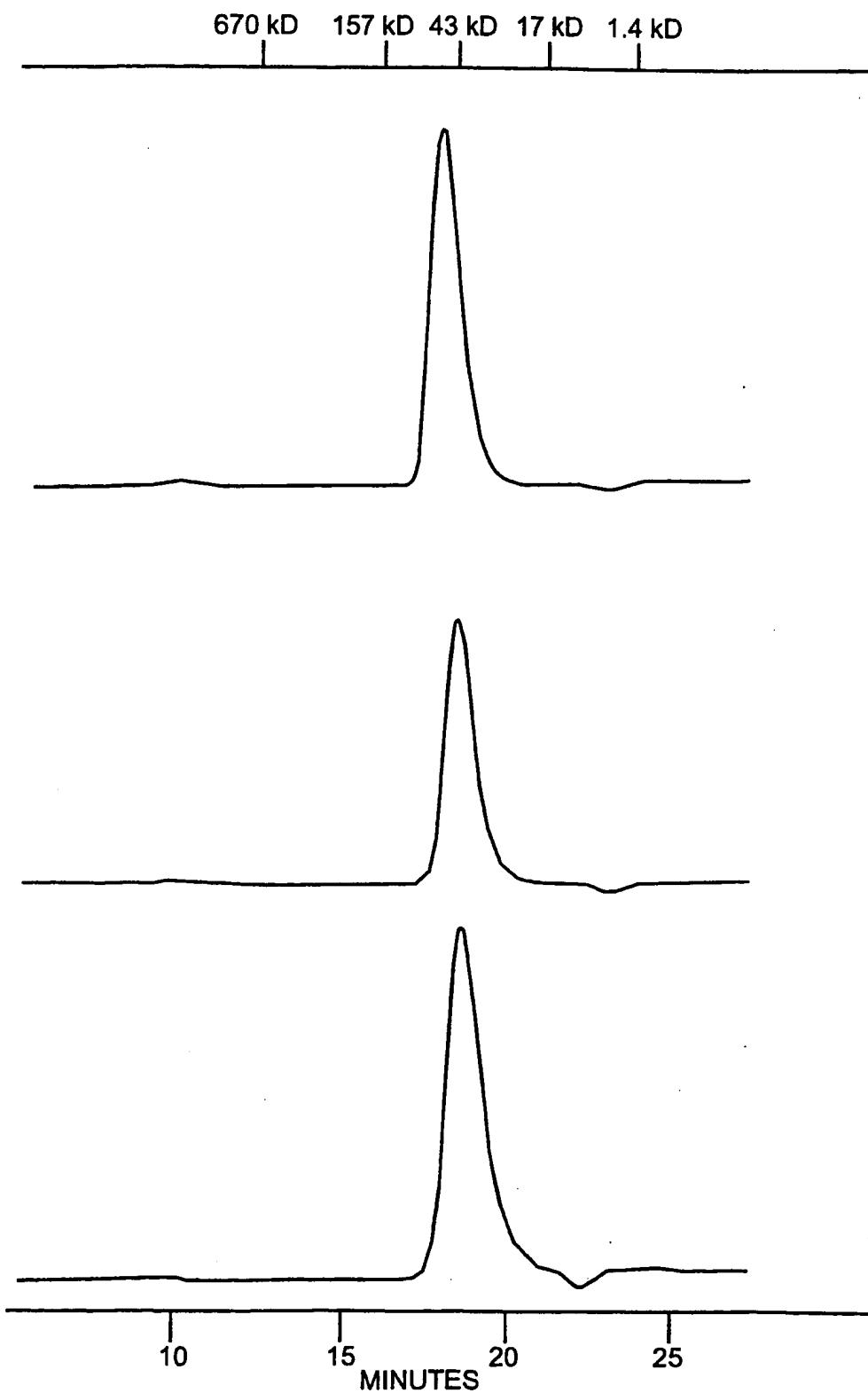


FIG. 4a

SUBSTITUTE SHEET (RULE 26)

5/12

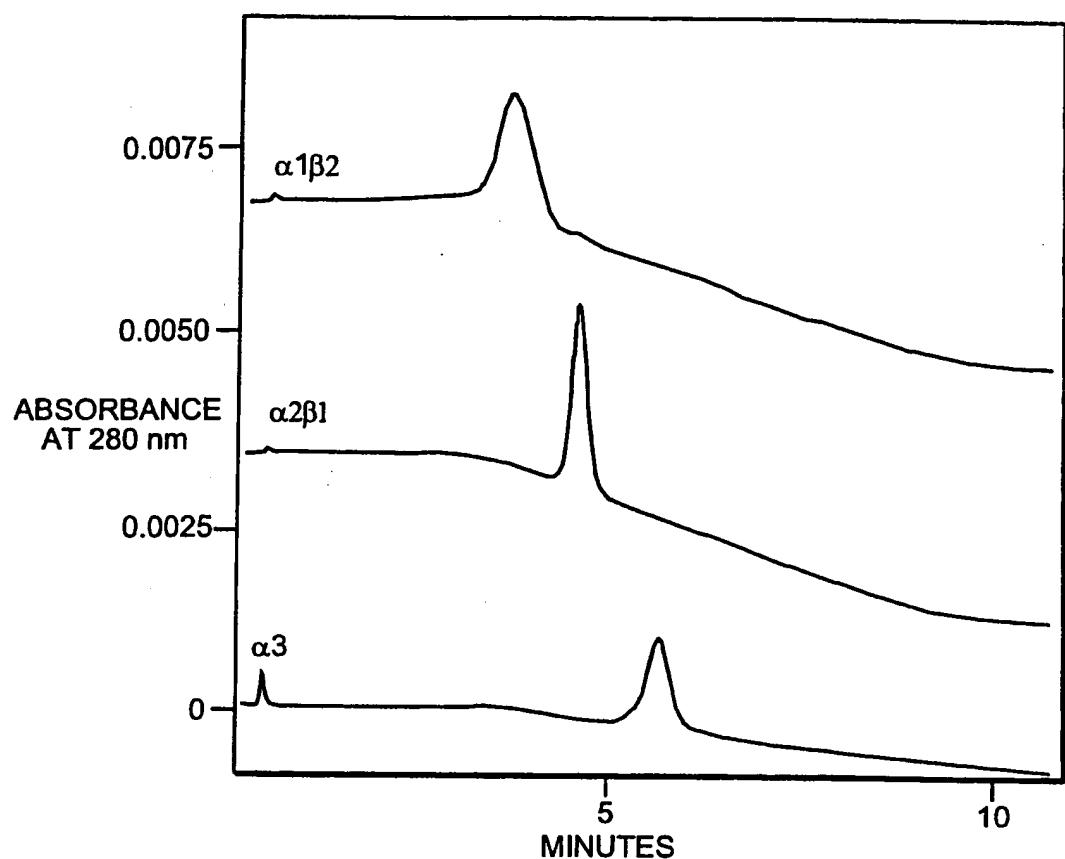


FIG. 4b

6/12

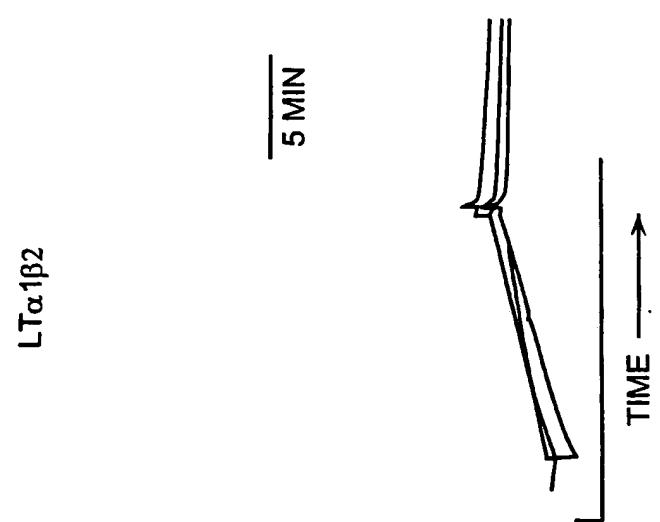


FIG. 5a-3

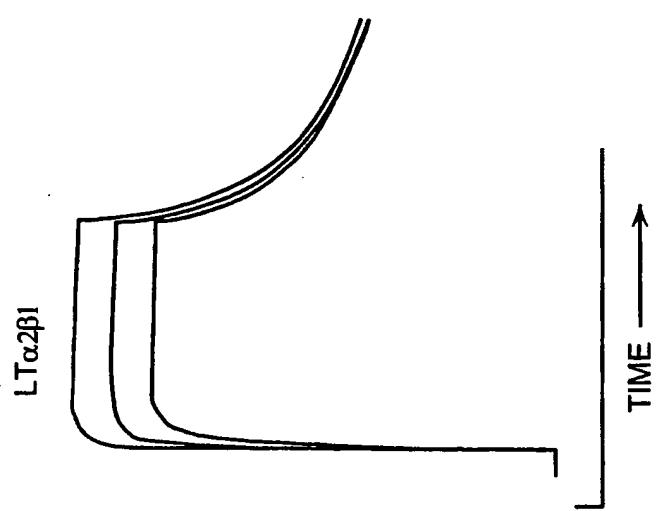


FIG. 5a-2

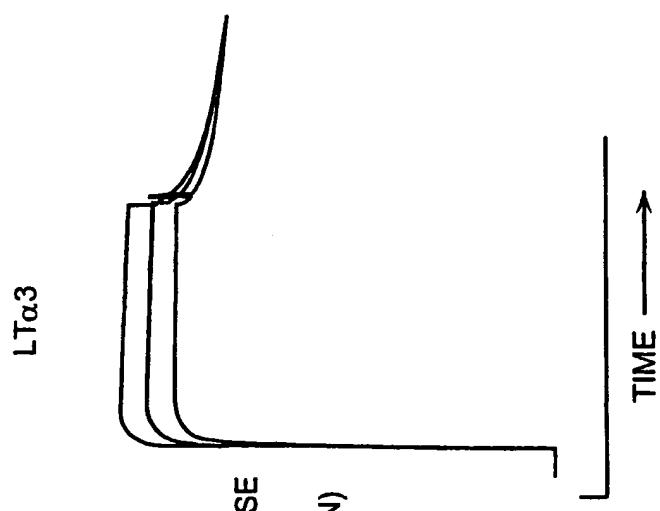


FIG. 5a-1

7/12

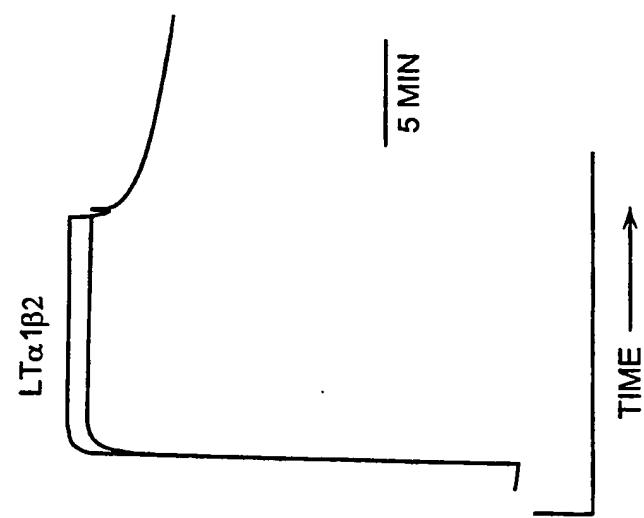


FIG. 5b-3

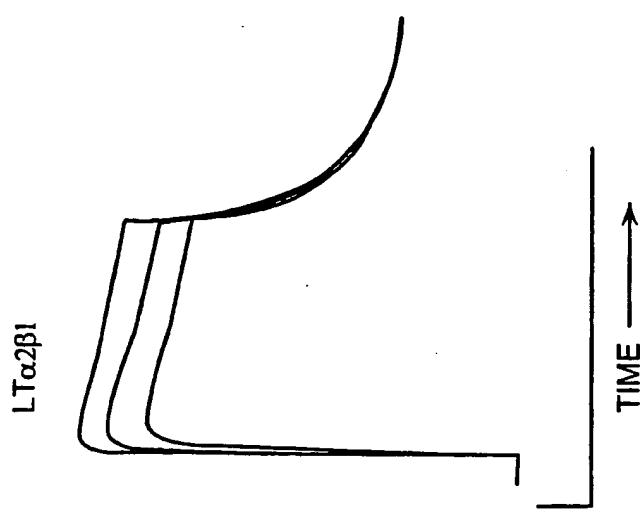


FIG. 5b-2

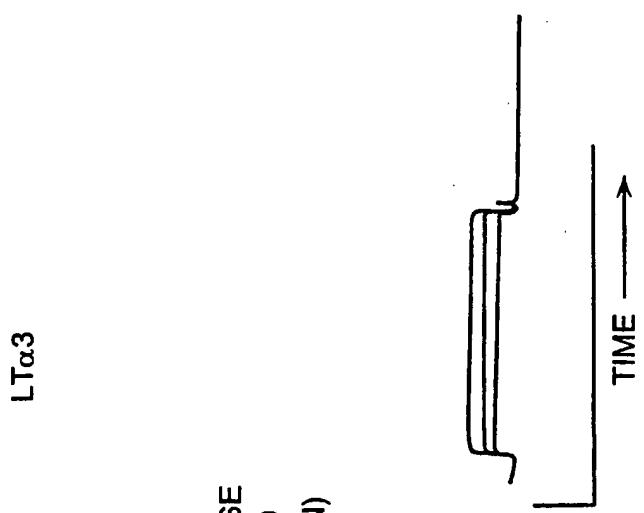


FIG. 5b-1

FIG. 6a

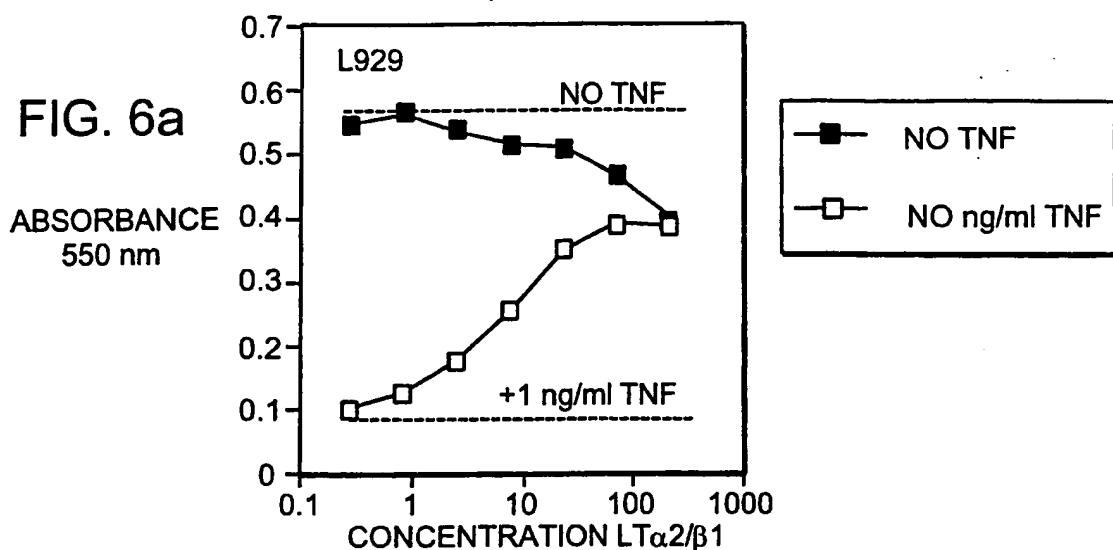


FIG. 6b

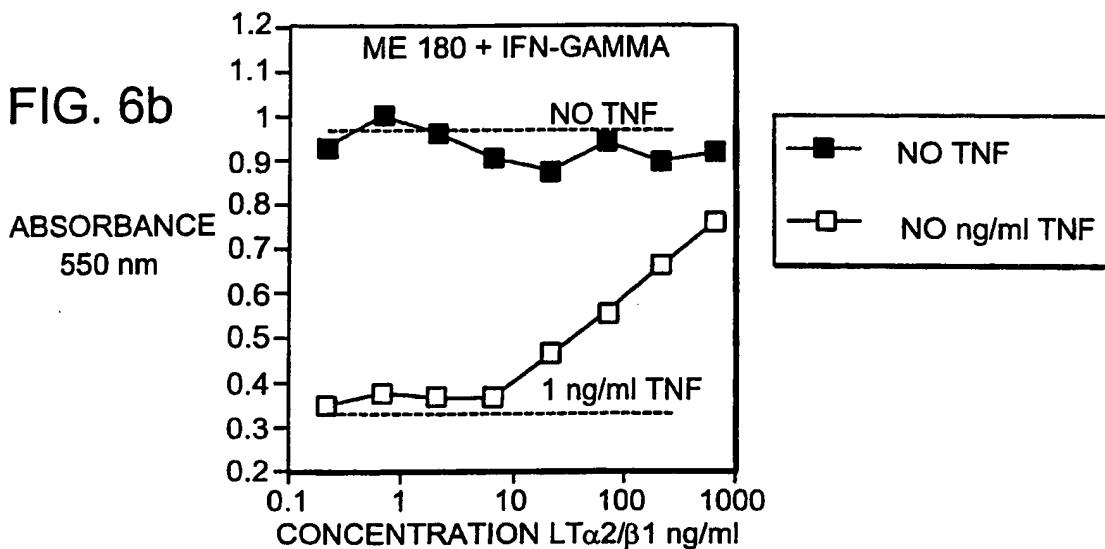
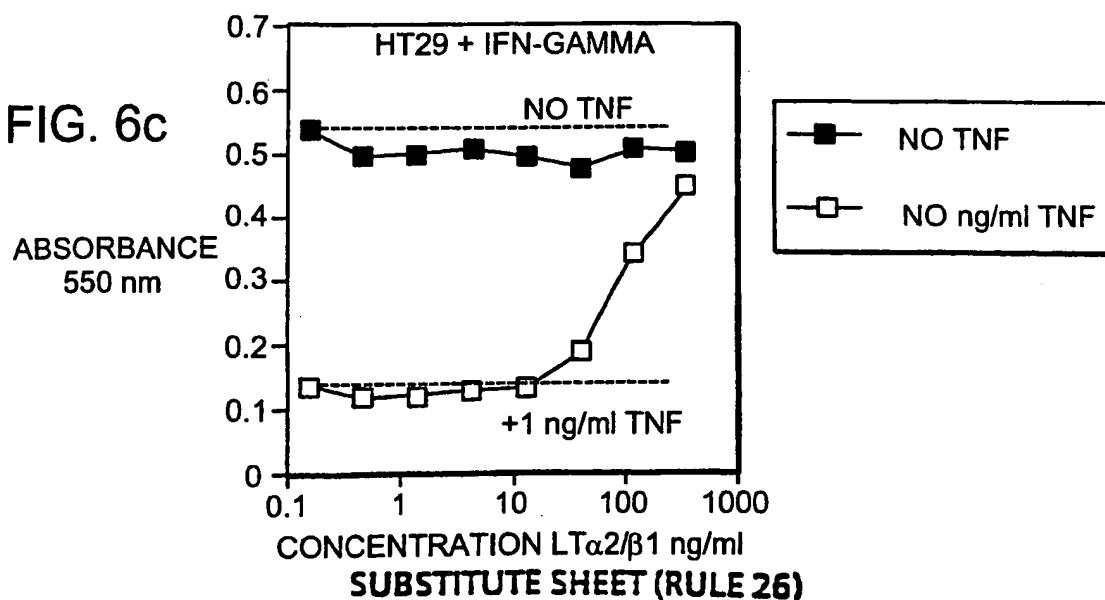


FIG. 6c



9/12

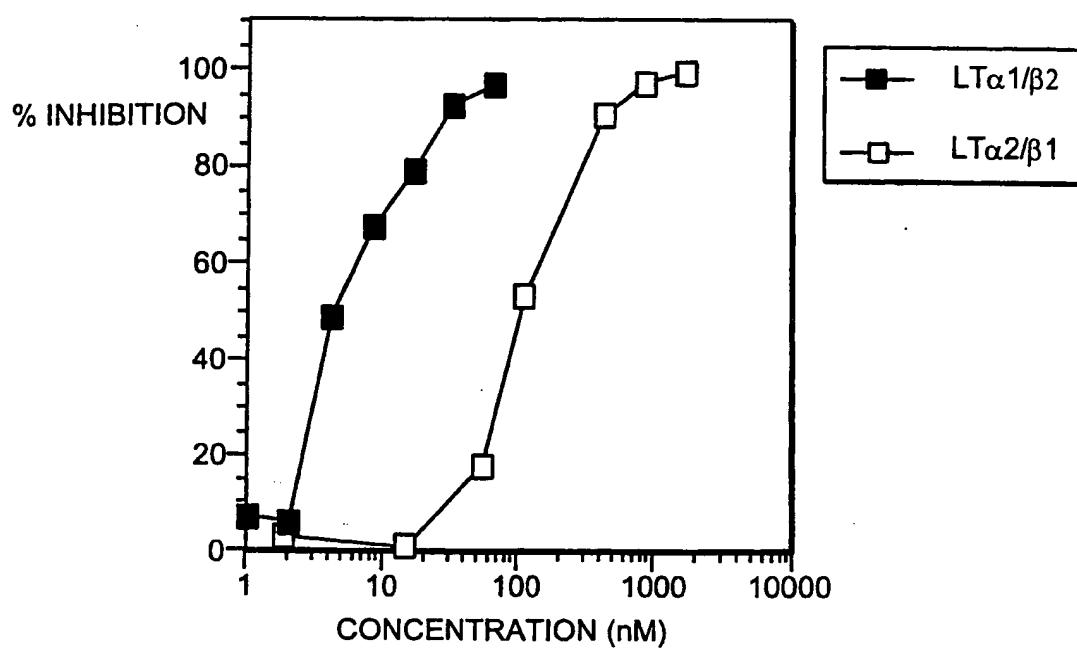


FIG.7

10/12

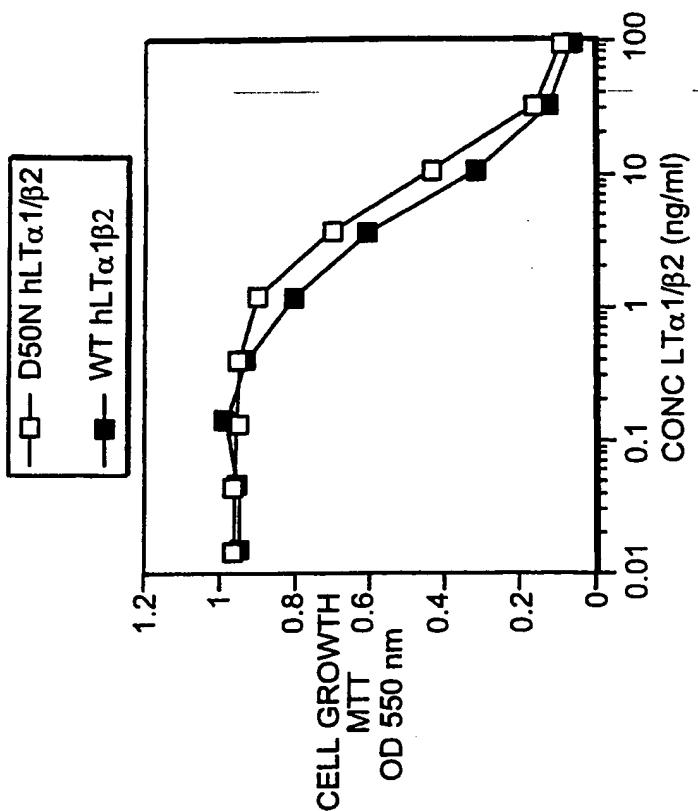


FIG. 8b

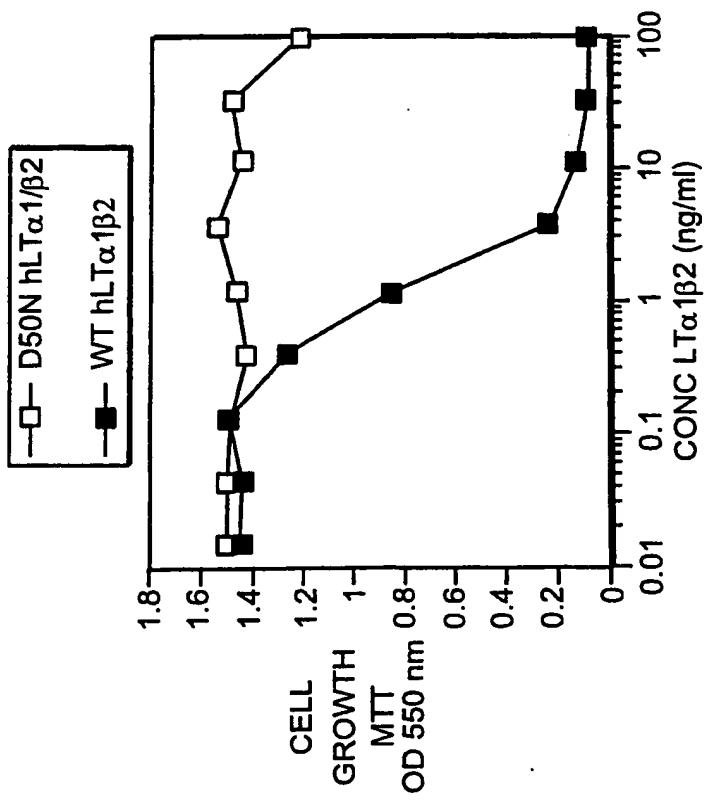


FIG. 8a

WO 96/40774

PCT/US96/09773

11/12

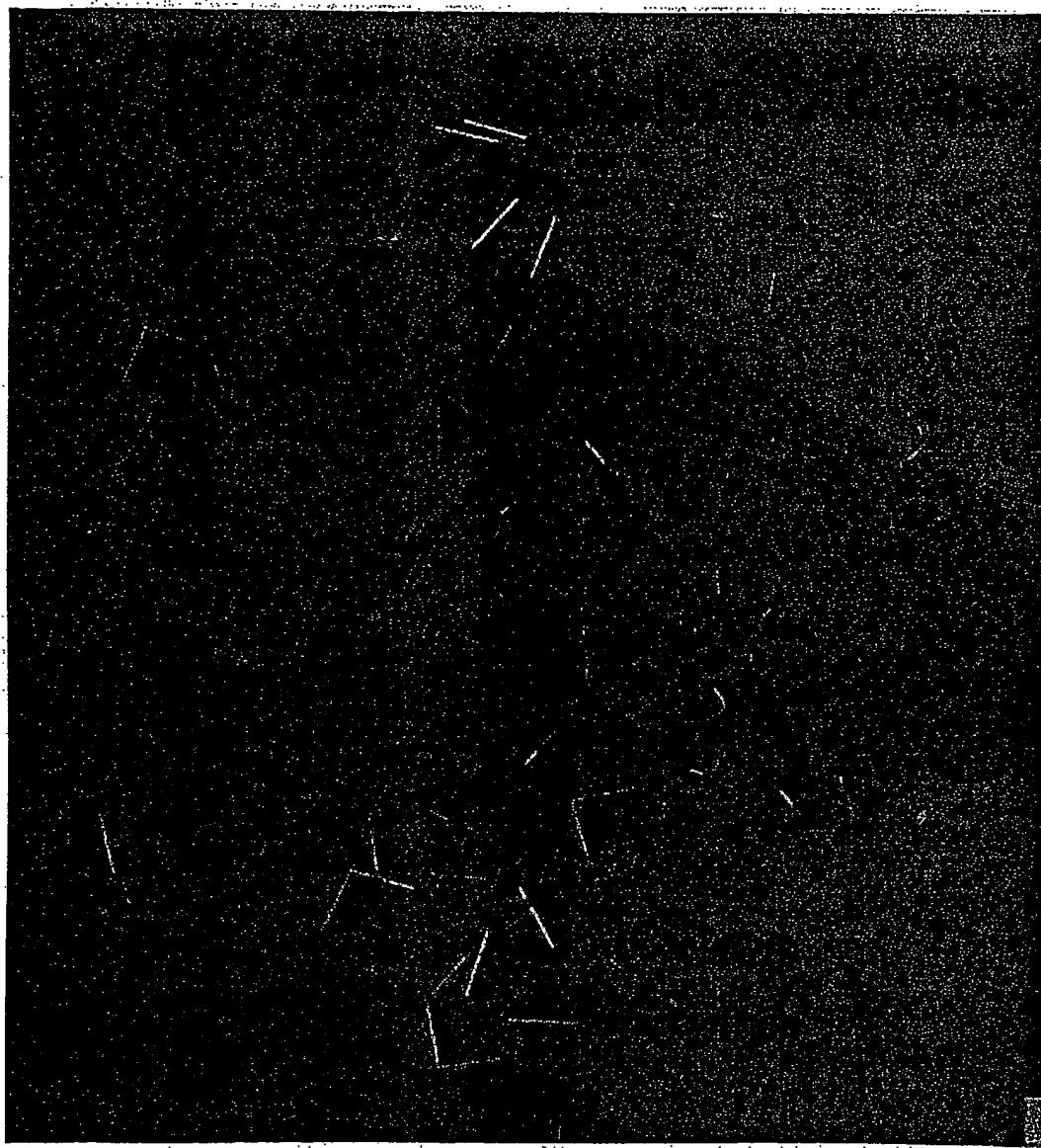


FIG. 9

SUBSTITUTE SHEET (RULE 26)

12/12

SEQ 1: CD40L/LT- $\alpha$  HYBRID

EEIKSQFEGF	VKDIMLNKEE	TKKENSFEMQ	KGDQNPQIAA	HVISEASSKT
TSVLQWAEGK	<u>YYFLQNNLV</u> T	LENGQLTVK	RQGLYFVYAQ	VVFCSNREAS
SQAPFIASLC	LKSPGRFERI	<u>LLSSOKTVYS</u>	AKPCGOHSIY	LGAAFELQPG
ASVFVNVTDP	SQVSHSTGFT	SFGA <u>FKL</u>		

SEQ 2: CD40L/LT- $\beta$  HYBRID

EEIKSQFEGF	VKDIMLNKEE	TKKENSFEMQ	KGDQNPQIAA	HVISEASSKT
TSVLQWAEGK	<u>YYFLTNNLV</u> T	LENGQLTVK	RQGLYLYAL	VGFCSNREAS
SQAPFIASLC	LKSPGRFERI	<u>LLEGAETVLS</u>	AKPCGOTSIG	LGGLFELQPG
ASVFVNVTDP	SQVSHSTGFT	SFGA <u>VKL</u>		

SEQ 3: FasL/LT- $\alpha$  HYBRID

QLFHLQKELA	ELRESTSQMH	TASSLEKQIG	HPSPPPEKKE	LRKA AHLTGK
SNSRSMPLEW	<u>EDTYGIVFLO</u>	GVKYKKGGLV	INETGLYFVY	<u>SOVVFRGQSC</u>
NNLPLSHKVY	MRNSKYPQDL	<u>VLLSSOKMVY</u>	CTTGQMWAHS	SYLGAAFNLT
TSADHLYVNV	SESLVNFEE	<u>STTFFGAFKL</u>		

SEQ 4: FasL/LT- $\beta$  HYBRID

QLFHLQKELA	ELRESTSQMH	TASSLEKQIG	HPSPPPEKKE	LRKA AHLTGK
SNSRSMPLEW	<u>EDTYGIVF</u> LT	GVKYKKGGLV	INETGLYLY	<u>SLVGFRGQSC</u>
NNLPLSHKVY	MRNSKYPQDL	<u>VLLEGAEMVL</u>	CTTGQMWATS	<u>SGLGGLFNLT</u>
TSADHLYVNV	SESLVNFEE	<u>GKTFFGAVKL</u>		

SEQ 5: TNF/LT- $\alpha$  HYBRID

VRSSSRTPSD	KPAAHVVANP	QAEGQLQWL	RRANAFLONG	VELRDNQLVV
PSEG <del>LYFVYS</del>	QVYFKGQGCP	STHVL	SRIAVSYQTK	VNLSSOKSP
CQRETPEGAE	AKPWYHSIYL	<u>GAAFQLEKGD</u>	RLSAEINRPD	YLDFAESTQV
<u>FFGAFAL</u>				

SEQ 6: TNF/LT- $\beta$  HYBRID

VRSSSRTPSD	KPAAHVVANP	QAEGQLQWL	RRANAFLTNG	VELRDNQLVV
PSEG <del>LYYLYS</del>	<u>LVGFKGQGCP</u>	STHVL	SRIAVSYQTK	<u>VNLLEGAESP</u>
CQRETPEGAE	AKPWYTSIGL	<u>GGLFQLEKGD</u>	RLSAEINRPD	YLDFAEGKQV
<u>FFGAVAL</u>				

FIG. 10  
SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/09773

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: 25-40

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 25-40

is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2.  Claims Nos.:

because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3.  Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International Application No
PCT/US 96/09773

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/525 C07K19/00 A61K38/19 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE, vol. 264, no. 5159, 29 April 1994, WASHINGTON, DC, USA, pages 707-710, XP002003806 P. CROWE ET AL.: "A lymphotoxin-beta-specific receptor." cited in the application see the whole document ---	1,2,4,5, 13-15, 18,19, 23-40
X	THE FASEB JOURNAL, vol. 8, no. 4, 15 March 1994, BETHESDA, MD, USA, page A509 XP002016898 P. CROWE ET AL.: "Production of soluble lymphotoxin (LT) alpha/beta mutein complexes using the baculovirus expression system." see abstract 2948 ---	1,2,4,5
		-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- '&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 October 1996	05.11.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax (+31-70) 340-3016	Authorized officer  Nooij, F

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/09773

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 13808 A (BIOPHARMA INC. ET AL.) 23 June 1994 cited in the application  see examples see claims ---	1,2,4,5, 23,25, 27,29, 31,33,37
X	WO 92 00329 A (BIOPHARMA INC. ET AL.) 9 January 1992 cited in the application  see examples see claims ---	1,2,4,5, 25,27, 29,31, 33,37
A	PROTEIN ENGINEERING, vol. 4, no. 7, October 1991, OXFORD, GB, pages 785-791, XP002016899 C. GOH ET AL.: "Aspartic acid 50 and tyrosine 108 are essential for receptor binding and cytotoxic activity of tumour necrosis factor beta (lymphotoxin)." cited in the application see abstract ---	6,7,17, 20
A	EUROPEAN CYTOKINE NETWORK, vol. 5, no. 6, November 1994, MONTROUGE, pages 83-96, XP000608365 K. WARZOWA ET AL.: "Mechanisms of action of the tumor necrosis factor and lymphotoxin ligand-receptor system." see the whole document ---	1-40
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 15, 12 April 1996, BALTIMORE, MD, USA, pages 8618-8626, XP002016900 J. BROWNING ET AL.: "Preparation and characterization of soluble recombinant heterotrimeric complexes of human lymphotoxins alpha and beta." see abstract ---	1,2,4-7, 13-19
P,X	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 183, no. 3, 1 March 1996, NEW YORK, NY, USA, pages 867-878, XP000608398 J. BROWNING ET AL.: "Signaling through the lymphotoxin beta receptor induces the death of some adenocarcinoma tumor lines." see abstract -----	1,2,5-7, 13-19, 23,24, 31,32

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/US 96/09773

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9413808	23-06-94	AU-A-	6048394	04-07-94
		CA-A-	2150249	23-06-94
		EP-A-	0672143	20-09-95
		JP-T-	8507201	06-08-96
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WO-A-9200329	09-01-92	AU-A-	2716795	15-02-96
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		CA-A-	2086264	28-12-91
		EP-A-	0536299	14-04-93
		JP-T-	6501456	17-02-94
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